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Signal Peptide-Dependent Protein Transport in *Bacillus subtilis*: a Genome-Based Survey of the Secretome

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GENERAL INTRODUCTION

A common feature in cells of prokaryotic and eukaryotic origin is the export of proteins from their site of synthesis, mostly the cytoplasm, to other destinations either inside or outside the cell. To achieve this, exported proteins are usually synthesized as precursors with an amino-terminal, transient "zip code" (signal peptide), which is recognized and deciphered by a cellular sorting and translocation machinery (318–320). Signal peptides consist of short stretches of amino acids which, after protein delivery to the correct subcellular compartment, are frequently removed by specialized signal peptidases. In general, a preprotein is first recognized by soluble targeting factors for its transport to the target membrane, where the protein becomes associated with a translocation machinery. Next, the polypeptide chain is transported through a proteinaceous channel. In most cases this transport process is driven by a translocation motor that binds and hydrolyzes

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nucleoside triphosphates. Finally, the signal peptide is removed, resulting in release of the mature protein from the translocase. If the protein is translocated in an unfolded conformation, the mature protein will fold into its native conformation shortly after release from the translocase. Notably, several integral membrane proteins retain their signal-like peptides and diffuse from the translocase laterally. These basic principles of protein transport across membranes apply to most eukaryotic and prokaryotic organisms (84, 216, 231, 249).

In eukaryotic cells, proteins can be transported to numerous destinations, such as the nucleus, the endoplasmic reticulum (ER), the Golgi apparatus, lysozomes, the plasma membrane, the cell wall, chloroplasts, mitochondria, peroxisomes, and the different membrane systems or compartments within the organelles mentioned. Furthermore, proteins can be secreted into the external environment of the cell. In contrast, in eubacterial and archaeal cells, protein sorting seems to be limited to a few compartments, such as the cytoplasmic membrane, the cell wall (gram-positive eubacteria and archaea), the periplasm, and the outer membrane (gram-negative eubacteria). In addition, eubacteria and archaea can secrete proteins directly into their growth medium. In order to do so, these unicellular organisms can exploit multiple pathways, such as the general secretory (Sec) pathway, the twin-arginine translocation (Tat) pathway, and ATP-binding cassette (ABC) transporters.

SCOPE OF THIS REVIEW—THE SECRETOME

In the following sections of this review, the known signal peptide-dependent protein transport pathways, as they are present in the gram-positive eubacterium *Bacillus subtilis*, will be discussed, with a strong focus on the Sec pathway. Notably, the protein export machineries of the gram-negative eubacterium *Escherichia coli* and certain eukarya, such as the yeast *Saccharomyces cerevisiae*, have in many cases been characterized in more detail than those of *B. subtilis*. For matters of comparison, these machineries will also be discussed where appropriate. Because of the differences in the cell envelope structure, differences in the machineries for protein export in *B. subtilis* and *E. coli* were anticipated more than a decade ago (205). As described in this review, such differences do indeed exist, especially at the early and late stages of protein export, making detailed characterization of the underlying molecular mechanisms a fascinating scientific challenge.

B. subtilis and related *Bacillus* species are well known for their industrial use in the production of secreted proteins. These eubacterial species are particularly attractive for this purpose because they have a high capacity to secrete proteins into the growth medium and because of their nonpathogenicity. Moreover, good fermentation technologies exist for various bacilli (36, 40, 41, 263). Many proteins can be secreted to very high levels by *B. subtilis*, such as the α-amylase AmyQ from *Bacillus amyloliquefaciens* (1 to 3 g/liter) (204), protein A from *Staphylococcus aureus* (>1 g/liter) (91), and human interleukin-3 (227). Although not precisely documented in the scientific literature, about 10-fold-higher secretion levels can be reached in optimized industrial fermentation systems using *Bacillus amyloliquefaciens* or *Bacillus licheniformis* strains. Unfortunately, the secretion of proteins of gram-negative eubacterial or eukaryotic origin by *Bacillus* species is often severely hampered due to several bottlenecks in the secretion pathway, such as poor targeting to the translocase, degradation of the secretory protein, and slow or incorrect folding. Therefore, it is not only of scientific but also of applied interest to define the so-called secretome of *B. subtilis*, which includes both the components of machineries for protein secretion and the native secreted proteins. In recent years, considerable progress has been made concerning the identification and characterization of host functions needed for protein secretion by *B. subtilis*. In particular, this progress was facilitated by the availability of the complete genome sequence of *B. subtilis* (149). Present research efforts on protein transport in *B. subtilis* are aimed first at obtaining a complete description of the secretome and second at identifying those secretome components that are limiting factors in secretion. This review will provide a first, largely genome-based survey of the secretome.

PROTEIN TRANSPORT IN *B. SUBTILIS*

At first sight, protein transport in *B. subtilis* appears to be a relatively simple process, as its cell structure is considerably less complicated than that of eukaryotic cells. The cytoplasm is surrounded by the cytoplasmic membrane, which is covered by a thick layer (10 to 50 nm) of peptidoglycan-containing anionic polymers, such as teichoic and teichuronic acid. All proteins of *B. subtilis* lacking transport signals will be retained in the cytoplasm and fold, with or without the aid of chaperones, such as GroEL-GroES and DnaK-DnaJ-GrpE, into their native conformation (16, 90, 111, 113, 193). Other proteins contain membrane-spanning domains that are required for their insertion into the cytoplasmic membrane.

Most proteins that are completely transported across the cytoplasmic membrane are synthesized with an amino-terminal signal peptide. As *B. subtilis*, like other gram-positive eubacteria, lacks an outer membrane, many of these proteins are secreted directly into the growth medium. In most cases, these secreted proteins are enzymes involved in the hydrolysis of natural polymers, such as proteases, lipases, carbohydrases, DNases, and RNases. Such degradative enzymes are frequently synthesized as part of an adaptive response to changes in the environment, allowing the cell to benefit optimally from the available resources (95, 177, 263). Subsequently, specialized uptake systems in the cytoplasmic membrane internalize (partially) degraded substrates (14, 104). A second well-described class of secreted proteins, consisting of seven relatively small proteins, denoted PhrA to PhrK, are used to sense the cell density of the population, thereby regulating the onset of post-exponential-phase processes, such as competence development and sporulation (152, 208). These Phr proteins are, after their secretion and processing into small peptides, reimported to fulfill their inhibitory action on certain cytoplasmic phosphatases (206, 207, 209, 271). In contrast to the degradative enzymes and Phr proteins, most other exported proteins, involved in processes such as cell wall turnover, substrate binding, or protein secretion (217, 281), have to be retained at the membrane-cell wall interface to fulfill their function. To prevent the loss of these proteins, they can contain signals for their attachment to the membrane (lipid modifications) or the cell wall. Alternatively, some exported proteins have the potential to form pilin-like structures at the membrane-cell wall interface.

Strikingly, under conditions of nutrient starvation, the formation of two genuine internal compartments, as encountered in organelles of the eukaryotic cell, is induced. These compartments, which ultimately develop into an endospore, are confined by two membranes, the forespore inner and outer membranes. The forespore inner membrane confines the cytosol of the forespore, while the forespore outer membrane forms the initial barrier between the forespore and the cytosol of the mother cell (89, 279) (for details, see the section on sporulation-specific protein transport). Recent data indicate that certain proteins are specifically sorted from the cytosol of the mother cell or the forespore to the intermembrane space (IMS)

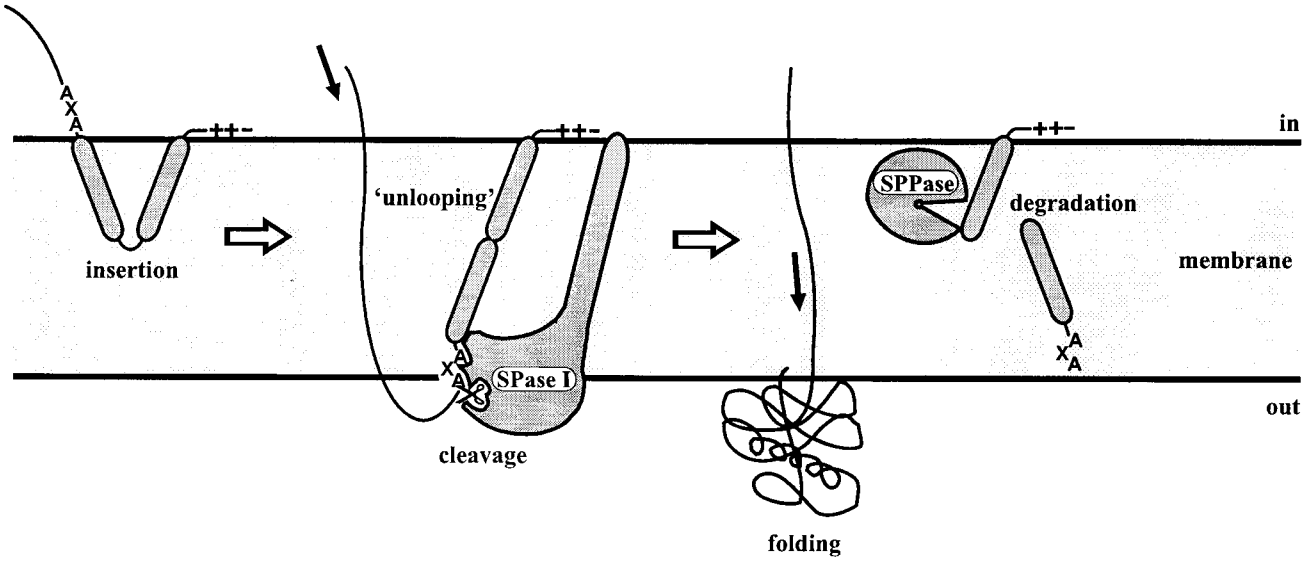

FIG. 1. Model for signal peptide insertion into the cytoplasmic membrane and cleavage by SPase I. First, the positively charged N-domain of the signal peptide interacts with negatively charged phospholipids in the membrane, after which the H-domain integrates loopwise into the membrane. Next, the H-domain unloops, whereby the first part of the mature protein is pulled through the membrane. During or shortly after translocation by a translocation machinery (not shown), the signal peptide is cleaved by SPase I and subsequently degraded by SPPases. After its translocation across the membrane, the mature protein folds into its native conformation.

between the two forespore membranes. The process of subcellular compartmentalization during sporulation in particular underscores the fact that, though simple at first glance, complex mechanisms for protein sorting have evolved in *B. subtilis*.

In the following sections, the amino-terminal cleavable signal peptides which are involved in the transport of ribosomally synthesized proteins in *B. subtilis* will be discussed. Furthermore, the different protein export routes and retention mechanisms which prevent the loss of certain exported proteins in the environment will be described.

Amino-Terminal Signal Peptides

Although the primary structures of different amino-terminal signal peptides show little similarity, three distinct domains can nevertheless be recognized (225, 316–319, 321). The amino-terminal N-domain of signal peptides contains at least one arginine or lysine residue, although this positively charged residue does not seem to be strictly required for protein export (51, 101). The positively charged N-domain has been suggested to interact with the translocation machinery (1) and negatively charged phospholipids in the lipid bilayer of the membrane during translocation (71). The H-domain, following the N-domain, is formed by a stretch of hydrophobic residues that seem to adopt an α-helical conformation in the membrane (37). Helix-breaking glycine or proline residues are frequently present in the middle of this hydrophobic core. The latter residues might allow the signal peptide to form a hairpin-like structure that can insert into the membrane. In one model for signal peptide function, it was proposed that unlooping of this hairpin results in insertion of the complete signal peptide in the membrane (71) (Fig. 1). Helix-breaking residues found at the end of the H-domain, are thought to facilitate cleavage by a specific signal peptidase (SPase) (67, 202). The C-domain, following the H-domain, contains the cleavage site for SPase, which removes the signal peptide from the mature part of the secreted protein during or shortly after translocation. The mature part of the protein is thereby released from the membrane and can fold into its native conformation. Finally, the signal peptide is degraded by signal peptide peptidases (SPPases) and removed from the membrane (Fig. 1). Although different amino-terminal signal peptides tend to be quite similar in general structure, apparently small differences between individual signal peptides can cause cleavage by different SPases, export via different pathways, and transport to different destinations.

Signal Peptide Classification

At present, four major classes of amino-terminal signal peptides can be distinguished on the basis of the SPase recognition sequence. The first class is composed of "typical" signal peptides which are present in preproteins that are cleaved by one of the various type I SPases of *B. subtilis* (289, 290, 292). Although most proteins having such a signal seem to be secreted into the extracellular environment, some of them are retained in the cell wall or sorted specifically to the IMS of endospores after membrane translocation via the Sec pathway. Notably, a subgroup of these signal peptides contain a so called twin-arginine motif (RR-motif), which might direct proteins into a distinct translocation pathway known as the Tat pathway.

The second major class of signal peptides is present in prelipoproteins, which are cleaved by the lipoprotein-specific (type II) SPase of *B. subtilis* (Lsp) (223, 291, 293). The major difference between signal peptides of lipoproteins and secretory proteins is the presence of a well-conserved lipobox in lipoprotein precursors. This lipobox contains an invariable cysteine residue that is lipid modified by the diacylglyceryl transferase prior to precursor cleavage by SPase II. After translocation across the cytoplasmic membrane, exported lipid-modified proteins remain anchored to the membrane by their amino-terminal lipid-modified cysteine residue (see the section on lipoprotein signal peptides for details). Notably, some signal peptides of lipoproteins contain a typical RR-motif. Consequently, the possibility exists that certain lipoproteins are exported via the Tat pathway rather than the Sec pathway.

The third major class is formed by signal peptides of prepilin-like proteins, which, in *B. subtilis*, are cleaved by the prepilin-specific SPase ComC (53). The recognition sequence for the prepilin SPase is, in contrast to that of secretory and lipoproteins, localized between the N- and H-domains, leaving

the H-domain attached to the mature pilin after cleavage (53, 54, 157, 225).

Finally, the fourth major class of signal peptides is found on ribosomally synthesized bacteriocins and pheromones that are exported by ABC transporters (11, 203, 335). These signal peptides lack a hydrophobic H-domain and are removed from the mature protein by a subunit of the ABC transporter that is responsible for the export of a particular bacteriocin or pheromone or by specific SPases.

HOW MANY PROTEINS ARE EXPORTED?

It is well established that *B. subtilis* can secrete certain proteins to high concentrations in the medium (184, 263). However, until recently it was difficult to estimate the number of exported proteins belonging to the secretome of *B. subtilis*. The completion of the *B. subtilis* genome sequencing project (149) and the availability of programs for the identification of signal peptides and transmembrane segments in large collections of protein sequences through worldwide web servers (194, 264) have now made it possible to predict the most likely location of all 4,107 annotated proteins (i.e., the proteome) of this organism. Computer-assisted studies have indicated that approximately 25% of the proteome of a given organism, such as *B. subtilis*, contains membrane sorting signals in the form of hydrophobic stretches of amino acids that can integrate in and span the membrane (35, 322; http://pedant.mips.biochem.mpg.de). Some of these putative membrane proteins contain amino-terminal signal peptides and may in fact be exported proteins, as indicated below.

Signal Peptide Predictions

To estimate the number of exported proteins, the amino termini of all annotated *B. subtilis* proteins in the SubtiList database (http://bioweb.pasteur.fr/GenoList/SubtiList) were used to predict amino-terminal signal peptides with the SignalP algorithm (194). This method incorporates a prediction of cleavage sites and a signal peptide/non-signal peptide prediction based on a combination of several artificial neural networks trained on the identification of signal peptides from gram-positive eubacteria. Next, all putative signal peptides were screened for the presence of a lipobox, RR-motif, or cleavage site for prepilin SPase. The numbers and features of each class of signal peptides are summarized in Fig. 2. It should be noted that polytopic membrane proteins, some of which can be cleaved by type I SPases (119, 288), were specifically excluded from the predictions, using the TopPred algorithm of Sipos and von Heijne (264). Furthermore, putative proteins with a single amino-terminal membrane-spanning domain, as encountered in certain type I SPases, might be falsely predicted to be secreted proteins. Finally, the neural networks of the SignalP algorithm, trained on data from gram-positive organisms, might not recognize some of the *B. subtilis* signal peptides with a more gram-negative or eukaryotic character.

Secretory (Sec-type) signal peptides. The signal peptide predictions resulted in the identification of 180 potential substrates for type I SPases. An RR-motif containing at least three residues of the R-R-X-#-# (where # is a hydrophobic residue) consensus sequence (18, 60) was found in 14 of these signals, suggesting that the corresponding preproteins are transported in a Sec-independent manner. The remaining 166 predicted "Sec-type" signal peptides (Table 1) had a length varying from 19 to 44 residues, with an average of 28 residues. These signal peptides contain on average two or three positively charged lysine (K) or arginine (R) residues in their N-domain, although some of the N-domains contain as many as 5 to 11 positively charged residues. The hydrophobic core (H-domain) has an average length of 19 residues, although a length of 17 or 18 residues seems to be preferred (Fig. 2 and 3). The C-domain of the predicted signal peptides carries a type I SPase cleavage site, with the consensus sequence A-X-A at position −3 to −1 relative to the SPase I cleavage site (Table 2). It is important to note that the C-domain must have an extended (β-sheeted) structure for efficient interaction with the active site of type I SPases. Based on the crystal structure of the type I SPase of *E. coli*, the side chains of residues at the −1 and −3 positions are thought to be bound in two shallow hydrophobic substrate-binding pockets (S1 and S3) of the active site, whereas the side chain of the residue at position −2 is pointing outwards from the enzyme (202). It is presumably for this reason that residues tolerated at positions −3 and −1 of the signal peptide are generally small and uncharged, while almost all residues (except cysteine and proline) seem to be allowed at position −2 (Table 2). Nevertheless, a preference for serine (18%) at position −2 of the signal peptide seems to exist in *B. subtilis*. According to the predictions, an alanine residue is most abundant (27%) at position +1 of the mature protein, but all other residues, with the exception of cysteine and proline, seem to be allowed at this position (Table 2). The absence of proline at the +1 position is consistent with the observation that the SPase I of *E. coli* was inhibited by recombinant preproteins with proline at this position (12, 196). Finally, approximately 60% of the predicted signal peptides contain a helix-breaking residue (mostly glycine) in the middle of the H-domain, and about 50% contain a helix-breaking residue (proline or glycine) at position −7 to −4 relative to the predicted processing site for SPase I.

Twin-arginine signal peptides. Proteins containing a signal peptide with the RR-motif (R-R-X-#-#, where # is a hydrophobic residue) may be transported via the Tat pathway. Through a database search for the presence of this motif in amino-terminal protein sequences, a total number of 27 putative RR-signal peptides were identified. Putative SPase I cleavage sites are present in 14 of these predicted signal peptides. These cleavage sites show no striking differences from those of the predicted Sec-type signal peptides (Fig. 2 and Table 3). Notably, the RR-motif was also found in the signal peptides of five putative lipoproteins, suggesting that these proteins might also be substrates for the Tat pathway (Table 4). Moreover, eight additional signal peptide-like sequences with the RR-motif but lacking cleavage sites for SPase I or SPase II were identified. The corresponding proteins have the potential to remain attached to the membrane with an amino-terminal transmembrane domain (Table 3). Interestingly, some of these proteins even contain additional transmembrane segments. Thus, the possibility exists that certain membrane proteins are translocated via the Tat pathway. Altogether, the N-domains of predicted RR-signal peptides of *B. subtilis* have an average length of 13 amino acid residues and are twice as long as the N-domains of the typical (Sec-type) signals. Strikingly, no significant differences are observed between the H-domains of predicted Sec-type and RR-signal peptides of *B. subtilis*. In contrast, it has been suggested that the H-domains of RR-signal peptides of *E. coli* are, on average, longer and less hydrophobic than those of Sec-type signal peptides of this gram-negative organism (60). These observations may suggest either that a difference in the H-domains of Sec-type and RR-signal peptides is not important for translocation via the Tat pathway in *B. subtilis* or that some of the predicted RR-signal peptides do not direct proteins into the Tat pathway. For example, it is conceivable that the latter possibility could apply to WapA and WprA, the secretion of which was impaired by Ffh or SecA depletion (119). Finally, positively charged residues (arginine or lysine) in the

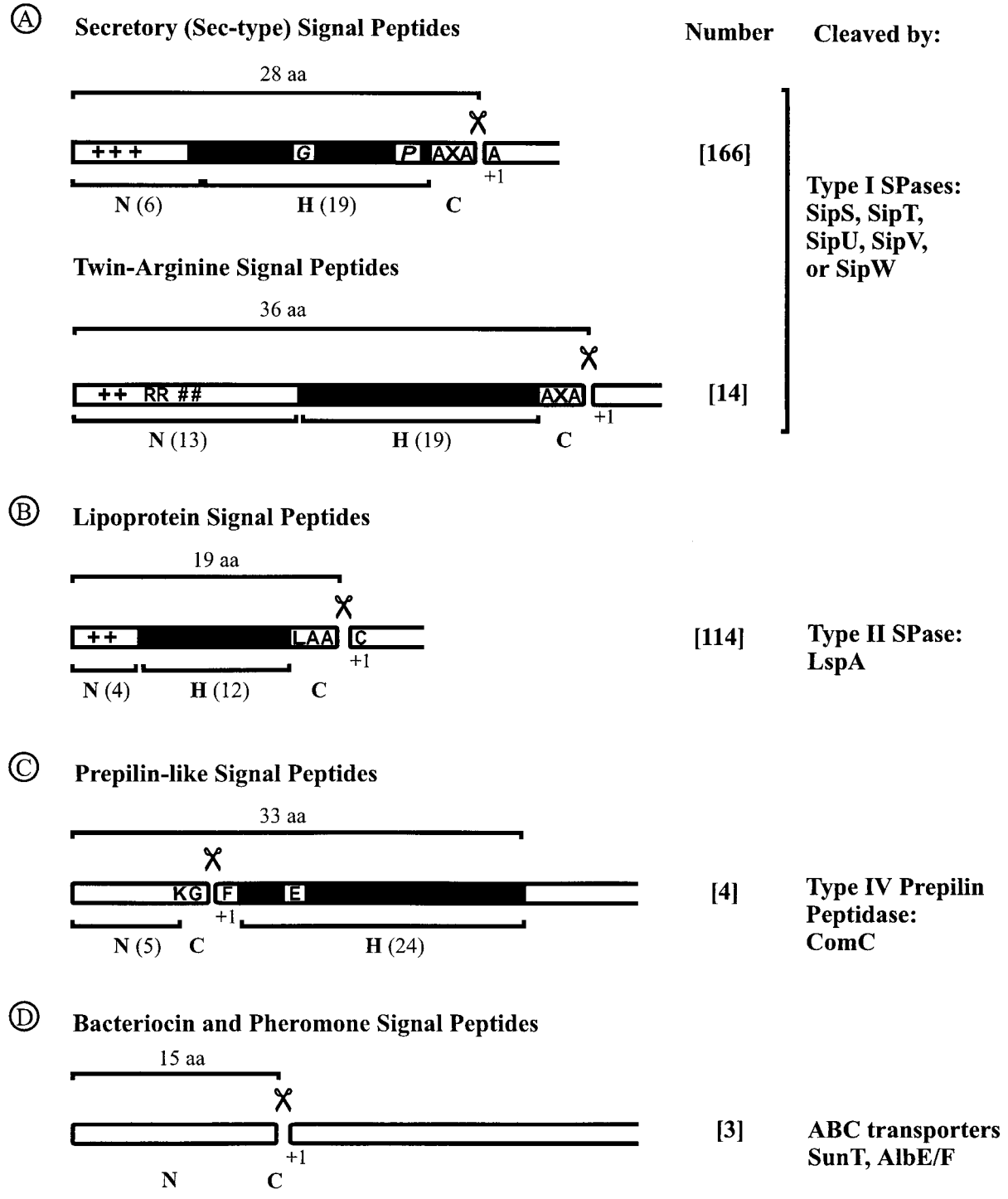

FIG. 2. Numbers and features of predicted amino-terminal signal peptides found in (putative) exported proteins of *B. subtilis*. To estimate the number of exported proteins, the first 60 residues of all annotated proteins of *B. subtilis* in the SubtiList database (http://bioweb.pasteur.fr/GenoList/SubtiList) were used to predict amino-terminal signal peptides with the SignalP algorithm for the prediction of signal peptides of gram-positive eubacteria (194). Next, to distinguish between potential secretory proteins and multispanning membrane proteins, putative membrane-spanning segments in protein sequences with a putative signal peptide were predicted with the TopPred2 algorithm (61, 264). All proteins containing additional hydrophobic domains (upper cutoff, 1.0; lower cutoff, 0.6; window size top, 11; window size bottom, 21) were regarded as membrane proteins, and their amino termini were excluded from the primary set of signal peptides. Finally, all putative signal peptides were screened for the presence of a lipobox, twin-arginine motif, or a cleavage site for the prepilin SPase. On the basis of SPase cleavage sites, predicted signal peptides were divided into four distinct classes: A, secretory (Sec-type) signal peptides and twin-arginine signal peptides; B, lipoprotein signal peptides; C, prepilin-like signal peptides; and D, bacteriocin and pheromone signal peptides. The number of predicted *B. subtilis* signal peptides of each class and the SPases responsible for their cleavage are indicated. Most signal peptides have a tripartite structure: a positively charged N-domain (N), containing lysine and/or arginine residues (indicated with +); a hydrophobic H-domain (H, indicated by a black box); and a C-domain (C) which specifies the cleavage site for SPase, as indicated by the scissors symbol. The average lengths of the complete signal peptide, N-domain, H-domain, and consensus SPase recognition sequences are indicated. Furthermore, helix-breaking residues, mostly glycine or proline (G/P) in the H-domain of certain signal peptides, are indicated. These residues are thought to facilitate loopwise membrane insertion and cleavage by SPase I, respectively (Fig. 1) (202). Finally, where appropriate, the most frequently occurring first amino acid (aa) of the mature protein (+1) is indicated.

TABLE 1. Predicted secretory (Sec-type) signal peptides of *B. subtilis*[a]

Protein	Signal Peptide	SPase I
AbnA[W]	MKKKKTWKRFLHFSSAALAAGLIFTSAAP	AEA AFW
AmyE	MFAKRFKTSLLPLFAGFLLLFHLVLAGPAA	ASA ETA
AprE	MRSKKLWISLLFALTLIFTMAFSNMS	VQA AGK
AspB	MKLAKRVSALTPSTTLAIT	AKA KEL
BglS	MPYLKRVLLLLVTGLFMSLFAVTAT	ASA QTG
BglC	MMRRRKRSDMKRSISIFITCLLITLLTMGGMIASP	ASA AGT
Bpr	MRKKTKNRLISSVLSTVVISSLLFPGAA	GA SSK
CccA	MKWNPLIPFLLIAVLGIGLTFFLS	VKG LDD
CitH	MGNTRKKVSVIGAGFTGATTAFLIAQKEL	ADV VLV
CotC	MKNRLFILICFCVICLFLSFGQPFFPSMILTVQA	AKS TRR
Csn	MKISMQKADFWKKAAISLLVFTMFFTLMMSET	VFA AGL
CwlD	MRKKLKWLSFLLGFIILLFLFKYQ	FSN NDS
DacB	MRIFKKAVFVIMISFLIATVNVNT	AHA AID
DacF	MKRLLSTLLIGIMLLTFAPS	AFA KQD
DltD	MKKRFFGPIILAFILFAG	AIA IPS
Epr	MKNMSCKLVVSVTLFFSFLTIGPL	AHA QNS
FliL	MKKKLMIILLIILIVIGALG	AAA YFV
FliZ	MKKSQYFIVFICFFVLFSVHPIAA	AAA DSD
GlpQ	MRKNRILALFVLSLGLLSFMVTP	VSA ASK
LipB	MKKVLMAFIICLSLILSVLAAPPSG	AKA ESV
LytB[W]	MKSCKQLIVCSLAAILLLIPSV	SFA ADS
LytC[W]	MRSYIKVLTMCFLGLILFVPT	ALA DNS
LytD[W]	MKKRLIAPMLLSAASLAFFAMSGS	AQA AAY
LytE[W]	MKKQIITATTAVVLGA	LFA HTS
LytF[W]	MKKKLAAGLTASAIVGTTLVVTP	AEA ATI
LytR	MRNERRKKKTLLLTILTIIGLLVLGTGGYAYYLWH	KAA STV
Mdr	MDTTTAKQASTKFVVLGLLLGILMSAMDNTIV	ATA MGN
MotB	MARKKKKKHEDEHVDESWLVPYADILTLLLALFIVLY	ASS SID
Mpr	MKLVPRFRKQWFAYLTVLCLALAAAVSFGVP	AKA AEN
MreC	MPNKRLMLLLLCIIILVAMI	GFS LKG
NprB	MRNLTKTSLLLAGLCTAAQMVFVTH	ASA EES
NprE	MGLGKKLSVAVAASFMSLSISLPG	VQA AEG
NucB	MKKWMAGLFLAAAVLLCLMVPQQIQG	ASS YDK
Pbp	MKKSIKLYVAVLLLFVVASVPYMHQA	ALA AEK
PbpB	MIQMPKKNKFMNRGAAILSICFALFFFVILG	RMA YIQ
PbpD	MTMLRKIIGWILLLCIIPLFAFT	VIA SGK
Pel	MKKVMLATALFLGLTPAG	ANA ADL
PelB	MKRLCLWFTVFSLFLVLLPGK	ALG AVD
PenP	MKLKTKASIKFGICVGLLCLSITGFTPFFNSTH	AEA KSI
PhoA	MKKMSLFQNMKSKLLPIAAVSVLTAGIF	AGA ELQ
PhoB	MKKFPKKLLPIAVLSSIAFSSLASGSVPE	ASA QEK
PhrA	MKSKWMSGLLLVAVGFSFTQVM	VHA GET
PhrC	MKLKSKLFVICLAAAAIFTAAGVS	ANA EAL
PhrF	MKLKSKLLLSCLALSTVFVAT	TIA NAP
PhrG	MKRFLIGAGVAAVILSGW	FIA DHQ
PhrK	MKKLVLCVSILAVILS	GVA LTQ
RpmG	MRKKITLACKTCGNRNYTTMKSS	ASA AER
SacB	MNIKKFAKQATVLTFTTALLAGGATQ	AFA KET
SacC	MKKRLIQVMIMFTLLLTMAFS	ADA ADS
SleB[W]	MKSKGSIMACLILFSFTITTFINTETIS	AFS NQV
SpoIID	MKQFAITLSVLCALILLVPTLLVIPFQHNKE	AGA SVE
SpoIIP	MRNKRRNRQIVVAVNGGKAVKAIFLFIVSLIVIFVL	SGV LTS
SpoIIQ	MREEEKKTSQVKKLQQFFRKRWVFPAIYLVSAAVILT	AVL WYQ
SpoIIR	MKKTVIICIYIFLLLSG	ALV GLA
TasA	MGMKKKLSLGVASAALGLALVGGG	TWA AFN
TyrA	MNQMKDTILLAGLGLIGGSI	ALA IKK

Continued on following page

TABLE 1—*Continued*

Protein	Signal Peptide	SPase I
Vpr	MKKGIIRFLLVSFVLFFALSTGITGVQAAPA	SSK
XynA	MFKFKKNFLVGLSAALMSISLFSATASA	AST
YbbC	MRKTIFAFLTGLMMFGTITAASA	SPD
YbbE	MKTKTLFIFSAILTLSIFAPNETFA	QTA
YbbR	MDKFLNNRWAVKIIALLFALLLYVAVNS	NQA
YbdG	MKTLWKVLKIVFVSLAALVLLVSVS	VFI
YbdN	MVKKWLIQFAVMLSVLSTFTYSASA	VGV
YbfO	MKRMIVRMTLPLLIVCLAFSSFSASARA	ASE
YbxI	MKKWIYVVLVLSIAGIGGFSVHA	ASS
YckD	MKRITINIITMFIAAAVISLTGTAEA	AEK
YdbK	MKLFNRKVTLVSLILMAVFQFFMALIIKRIVIS	AGT
YddT	MRKKRVITCVMAASLTLGSLLPAGYASA	KED
YdhT	MFKKHTISLLIIFLLASAVLA	KPI
YdjM	MLKKVILAAFILVGSTLGAFSFSSDASA	KHV
YdjN	MKKRIILLLAVIIAAAAAGVA	FYV
YfhK	MKKKQVMLALTAAAGLGLTALHSAPAAKA	APL
YfjS	MKWMCSICCAAVLLAGGAAQA	EAV
YfkD	MMKKLFHSTLIVLLFFSFFGVQPIHA	KKQ
YhaK	MRTWKRIPKTTMLISLVSPFLLITPVLFYAALA	FPN
YhdC	MKSLPYTIALLFCGLIIVSMA	AKG
YhfM	MKKIVAAIVVIGLVFIAFFYLYSRSGDVYQSVDA	DLI
YhjA	MKKAAAVLLSLGLVFGFSYGAGHVAEA	KTK
YjcM	MKKELLASLVLCLSLSPLVSTNEVFA	ATT
YjcN	MKKKTKIILSLLAALIVILIVLPVLSPVVFTASS	EKG
YjdB	MNFKKTVVSALSISALALSVSGVASA	HEI
YjfA	MKRLFMKASLVLFAVVFVFAVKGAPAKA	ETH
YjiA	MAAQTDYKKQVVGILLSLAFVLFVFS	FSE
YknX	MKKVWIGIGIAVIVALFVGINIYRSAAPTSGSA	GKE
YkoJ	MLKKKWMVGLLAGCLAAGGFSYNAFA	TEN
YkvT	MTTKFTALAVFLLCFMPAAKI	EHT
YkvV	MLTKRLLTIYIMLLGLIAWFPGAAQA	EEK
YkwD	MKKAFILSAAAAVGLFTFGGVQQASA	KEL
YlaE	MKKTFVKKAMLTTAAMTSAALLTFGPDAASA	KTP
YlbL	MLRKKHFSWMLVILILIAVLSFIKLPYYITKPGEA	TEL
YlqB	MKKIGLLFMLCLAALFTIGFPAQQADA	AEA
YlxF	MSGKKKESGKFRSVLLIIILPLMFLLIAGGIVLWAAG	INV
YlxW	MRGKSAVLLSLIMLIAGFLISFSFQMTKENNKSAA	ETE
YlxY	MYKKFVPFAVFLFLFFVSFEMMENPHALDYIGA	MKK
YncM	MAKPLSKGGILVKKVLIAGAVGTAVLFGTLSSGIPGLPAADA	QVA
YndA	MRFTKVVGFLSVLGLAAVFPLTAQA	EKA
YnfF	MIPRIKKTICVLLVCFTMLSVMLGPGATEVLA	ASD
YngK	MKVCQKSIVRFLVSLIIGTFVISVPFMANA	QSD
YnzA	MELSFTKILVILFVGFLVFGPDKLPALG	RAA
YoaW	MKKMLMLAFTFLLALTIHVGEASA	VIV
YobB	MKIRKILLSSALSFGMLISAVPALA	AGT
YobV	MKLERLLAMVVLLISKKQVQA	AEL
YocA	MKKKRKGCFAAAGFMMIFVFVIA	SFL
YocH[W]	MKKTIMSFVAVAALSTTAFGAHA	SAK
YodV	MKVPKTMLLSTAAGLLLSLTATSVSA	HYV
YojL[W]	MKKKIVAGLAVSAVVGSSMAAAPAEA	KTI
YolA	MKKRITYSLLALLAVVAFAFTDSSKAKA	AEA
YolC	MKKRLIGFLVLVPALIMSGITLIEA	NKK
YolI	MKKWIVLFLVLIAAAISIFVYVSTGSE	KPF
YomL	MRKKRVITCVMAASLTLGSLLPAGYATA	KED
YopL	MKKLIMALVILGALGTSYISA	DSS
YoqH	MKRFILVLSFLSIIVAYPIQTNA	SPM
YoqM	MKLRKVLTGSVLSLGLLVSASPAFA	TSP

Continued on following page

TABLE 1—*Continued*

Protein	Signal Peptide	SPase I
YpbG	MKLSVKIAGVLTVAAAAMTAKMY	ATA KGN
YpcP	MNNNKLLLVDGMALLFRAFF	ATA VHR
YpjP	MKLWMRKTLVVLFTIVTFGLVSPPAA	LMA DKP
YpmB	MRKKALIFTVIFGIIFLAVLLVSASIYKS	AMA QKE
YpmS	MNKWKRLFFILLAINFILAAGFVALVLLPGEQ	AQV KDA
YpuA	MKKIWIGMLAAAVLLLMVPKVSL	ADA AVG
YpuD	MGRIKTKITILLVLLLLLAGGYMYINDIELKDVPT	AIG QTL
YqfZ	MKRLTLVCSIVFILFILFYDLKIGTIPIQDLPVYE	ASA KTA
YqgA	MKQGKFSVFLILLLMLTLVVAPKGK	AEA ASS
YqxI	MFKKLLLATSALTFSLSLVLPLDGH	AKA QEV
YqxM	MFRLFHNQQKAKTKLKVLLIFQLSVIFSLTAAICLQFSDD	TSA AFH
YqzC	MTKRGIQAFAGGIILATAVLAAVFYLTDEDQ	AAA VKD
YqzG	MMIKQCVICLSLLVFGTTA	AHA EET
YraJ	MTLTKLKMLSMLTVMIASLFIFSSQ	ALA VQY
YrrL	MYINQQKKSFFNKKRIILSSIVVLFLIIGG	AFL YGK
YrrR	MKISKRMKLAVIAFLIVFFLLLLRL	AEI QLF
YrrS	MSNNQSRYENRDKRRKANLVLNILIAIVSILIVV	VAA NLF
YrvJ	MNKKYFVLIVCIIFTSALFPTFSS	VTA AQG
YuaB	MKRKLLSSLAISALSLGLLVSAPTASF	AAE STS
YunA	MITDIFKPGCRKLCVFNMKGDYFVKVLLSALLLLLFA	FEP
YunB	MPRYRGPFRKRGPLPFRYVMLLSVVFFILSTT	VSL WMI
YurI	MTKKAWFLPLVCVLLISGWLAPAAS	ASA QTT
YusW	MHLIRAAGAVCLAVVLIAGCRFNEDQHQ	AEG ENT
YvbX	MKKWLIIAVSLAIAIVLFMYTKGE	AKA AGM
YvcE[W]	MRKSLITLGLASVIGTSSFLIPFTSKT	ASA ETL
YveB	MNYIKAGKWLTVFLTFLGILLF	IDL FPK
YvgO	MKRIRIPMTLALGAALTIAPLSF	ASA EEN
YvgV	MKKKQQSSAKFAVILTVVVVVLLA	AIV IIN
YvnB	MRKYTVIASILLSFLSVL	SGG HHE
YvpA	MKKIVSILFMFGLVMGFSQFQPST	VFA ADK
YvpB	MKTLRTLCVLMILSGVIFFGLK	IDA KDI
YwaD	MKKLLTVMTMAVLTAGTLLLPAQSVTPA	AHA VQI
YwcI	MKRLLVSLRVWMVFLMNWVTPDRKT	ARA AVY
YwdK	MKVFIILGAINALLAVGLG	AFG AHG
YwfM	MKGNIYSLFVLIAAFFWGTTGT	VQA LAP
YwgB	MKMKSGMEQAVSVLLLLSRLP	VQA SLT
YwjE	MKVFIVIMIIVVIFFALILLDIFM	GRA GYR
YwmB	MKKKQVSHAIIISVMLSFVIAVFHT	IHA SEL
YwmC	MKKRFSLIMMTGLLFGLTSP	AFA AEK
YwmD	MKKLLAAGIIGLLTVSIASP	SFA AEK
YwoF	MRKWYFILLAGVLTSVILAFVYDK	TKA NEE
YwqC	MGESTSLKEILSTLTKRILLIMIVTAA	ATA AGG
YwqO	MKFLLSVIAGLLILALYLFWKVQPP	VWI QVE
YwsB	MNKPTKLFSTLALAAGMTAAAAGGAGT	IHA QQP
YwtC	MKFVKAIWPFVAVAIVFMF	MSA FKF
YwtD[W]	MNTLANWKKFLLVAVIICFLVPIMTKAEI	AEA DTS
YwtF	MEERSQRRKKKRKLKKWVKVVAGLMAFLVIAAGSVG	AYA FVK
YxaK	MVKSFRMKALIAGAAVAAAVSAGAVSDVPAAKVLQPTA	AYA AET
YxiA	MFNRLFRVCFLAALIMAFTLPNS	VYA QKP
YxiT	MKWNNMLKAAGIAVLLFSVFAYAAPSLKA	VQA KTP
YyaB	MVYQTKRDVPVTLMIVFLILLIQ	ADA IVP
YybN	MNKFLKSNFRFLLAAALGISLLASSNF	IKA SND
YycP	MKKWMITIAMLILAGIALFVFISP	LKS HKT

[a] Putative signal peptides were identified as described in the text under the heading Signal Peptide Predictions. Positively charged lysine (K) and arginine (R) residues in the N-domain are indicated in bold letters. The hydrophobic H-domain is indicated in gray shading. The residues at positions −3 to −1 relative to the predicted SPase I cleavage site are underlined, and the SPase cleavage site is indicated with a gap in the amino acid sequence. Proteins containing additional cell wall-binding repeats in the mature part of the protein are indicated with a superscript W.

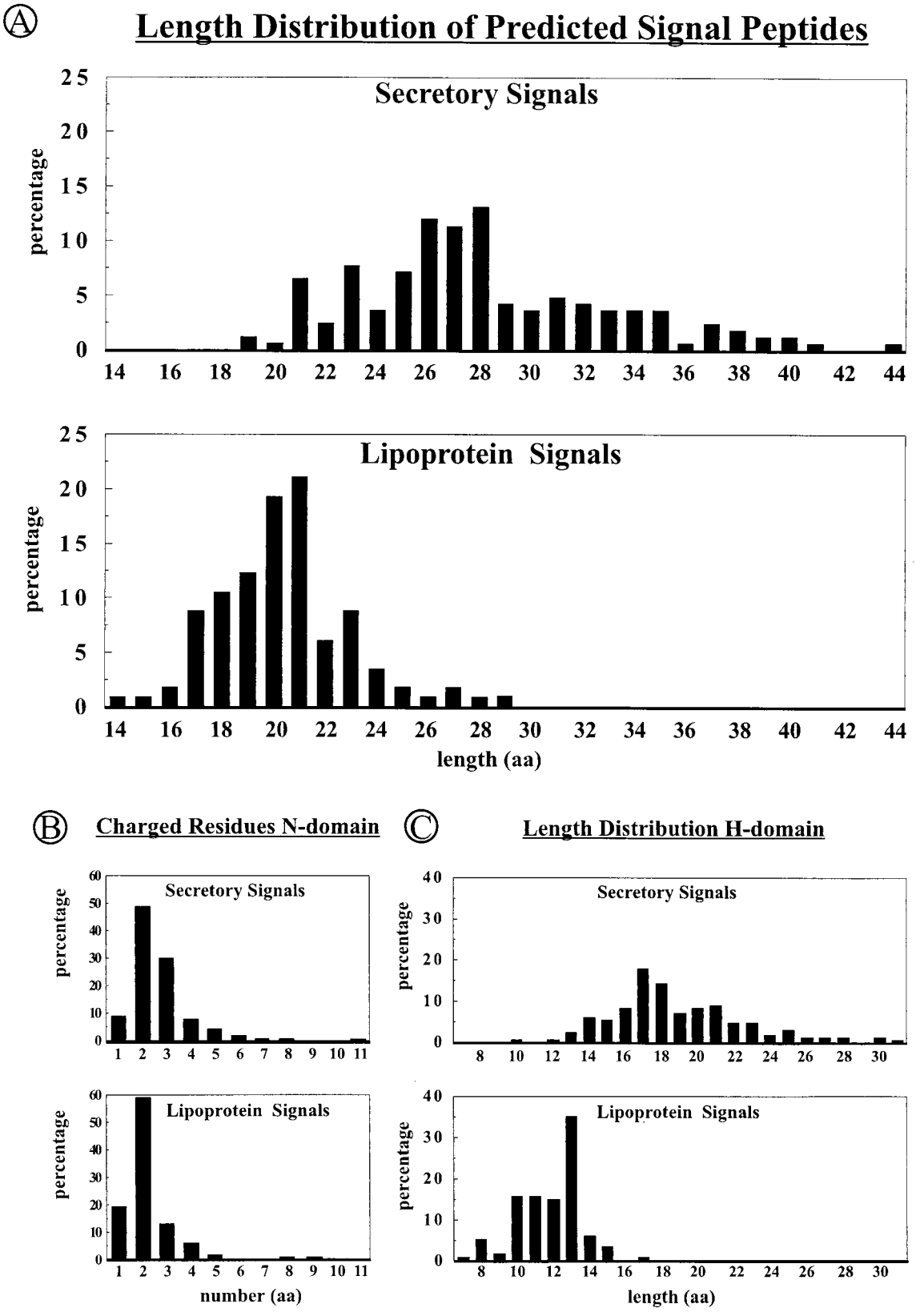

FIG. 3. Features of predicted secretory and lipoprotein signal peptides. (A) Length distribution of complete signal peptides (N-, H-, and C-domains). (B) Distribution of positively charged lysine or arginine residues in the N-domains of predicted signal peptides. (C) Length distribution of the hydrophobic H-domains in predicted signal peptides. Distributions are indicated as percentages of the total number of predicted secretory or lipoprotein signal peptides.

TABLE 2. Amino acid residues around (putative) SPase I cleavage sites[a]

Position and residue	Frequency (% of total)
−3	
A	60
V	18
I	6
S	5
G	5
T	3
L	2
F	1
Q	<1
M	<1
K	<1
Y	<1
W	<1
−2	
S	18
F	11
K	10
E	8
L	8
Q	7
A	7
H	6
I	5
D	5
T	4
G	4
N	4
V	3
M	2
R	2
Y	2
W	1
−1	
A	85
S	6
G	5
V	4
L	3
I	2
E	1
N	<1
+1	
A	27
K	10
Q	10
S	8
V	5
F	5
D	4
N	4
Y	3
L	4
I	4
H	3
G	2
T	2
E	1
R	1
W	1
M	<1

[a] The frequency of a particular amino acid at each position is given as the percentage of the total number of predicted signal peptides in which it appears.

C-domain, which can function as a so-called Sec avoidance signal that prevents interactions with Sec pathway components (23), are present in 8 of the 27 putative twin-arginine signal peptides.

Lipoprotein signal peptides. As the signal peptides of lipoproteins are, in general, shorter than those of secretory proteins, not all lipoproteins are recognized by the SignalP algorithm (194; our unpublished observations). In addition, some lipoproteins, such as CtaC (17) (Table 4), contain multiple membrane-spanning segments that were excluded from predictions of the signal peptides of secretory proteins mentioned above. Therefore, additional putative lipoprotein signal peptides were identified through similarity searches in the SubtiList database with signal peptides of known lipoproteins, using the Blast algorithm (4). Putative lipoprotein sorting signals identified by the latter method were combined with those identified by SignalP, resulting in a total number of 114 (Table 4). Signal peptides from lipoproteins differ in several respects from those of secretory signals. First, the structural features of lipoprotein signal peptides are more conserved than those of secretory signal peptides. This suggests that less variation in these peptides is allowed by the components involved in lipid modification and processing of lipoproteins. The C-domain contains a so-called lipobox with the consensus sequence L-(A/S)-(A/G)-C (Table 5), of which the invariable cysteine residue is the target for lipid modification and becomes the first residue of the mature lipoprotein after cleavage by SPase II. Second, both the N-domain (average of four residues) and the H-domain (average of 12 residues) seem, on average, to be shorter than the corresponding domains of signal peptides of nonlipoproteins (Fig. 2 and 3). Finally, helix-breaking residues are less abundant (27%) in the H-domain of lipoprotein signal peptides than in the corresponding regions of nonlipoprotein signal peptides. As transmembrane helices seem to require at least 14 hydrophobic residues to span the membrane (34, 46), the latter findings suggest that not all lipoprotein signal peptides can span the membrane completely. This implies that the active site of SPase II may be embedded in the cytoplasmic membrane, as was suggested for SPase I (202, 309). Alternatively, the N-domain of the signal peptide may not stay fixed at the cytoplasmic surface of the membrane during translocation. Strikingly, aspartic acid was absent from the +2 position of predicted mature lipoproteins. In mature lipoproteins of gram-negative eubacteria, an aspartic acid residue at the +2 position specifically prevents the sorting of these proteins to the outer membrane (167, 168, 221). The fact that aspartic acid at the +2 position is absent from *Bacillus* lipoproteins suggests that this sorting signal has evolved exclusively in gram-negative eubacteria. Nevertheless, it has to be noted that glycine, phenylalanine, or tryptophan residues can be found at the +2 position of various lipoproteins of *B. subtilis* (Table 5). Like aspartic acid, these residues prevent lipoprotein sorting to the outer membrane of *E. coli* (260). Thus, even though *B. subtilis* lacks an outer membrane, residues with a potential sorting function can be found at the +2 position of mature lipoproteins of *B. subtilis*. It is therefore conceivable that such residues are involved in the targeting of lipoproteins to specific membrane locations in *B. subtilis*. Nevertheless, it has to be emphasized that presently no experimental data are available to support this idea.

Type IV prepilin signal peptides. Only four proteins required for DNA binding and uptake during competence (ComGC, ComGE, ComGD, and ComGG), which are already known to contain type IV prepilin-like signal peptides (55), were identified in our SignalP and Blast homology searches for prepilin-like signal peptides in *B. subtilis* (Table 6). As the prepilin SPase (ComC) acts at the cytoplasmic side of the membrane (151, 157), the "C-domain" with the ComC cleav-

TABLE 3. Predicted twin-arginine signal peptides[a]

Protein	RR-motif	Signal Peptide
AlbB	MSPAQ **RRILL**	YILSFIFVIGAVVYFVKSDYLFTLIFIAIA ILF
AmyX TM	MVSI **RRSFE**	AYVDDMNIITVLIPAEQ***K***EIM
AppB TM	MAAYII **RRTLM**	SIPILLGITILSFVIM***K***AAPG
LipA	MKFVK **RRIIA**	LVTILMLSVTSLFALQPSA***K***A AEH
OppB TM	MLKYIG **RRLVY**	MIITLFVIVTVTFFLMQAAPG
PbpX	MTSPTRTAKR **RRRKL**	NKRGKLLFGLLAVMVCITIWNA LHR
PhoD	MAYDSRFDEWVQKLKEESFQNNTFD **RRKFI**	QGAGKIAGLSLGLTIAQSVGA FEV
QcrA TM	MGGKHDIS **RRQFL**	NYTLTGVGGFMAASMLMPMV***R***FA
TlpA TM	MKKTLTTIRRSSIA **RRLII**	SFLLILIVPITALSVSAYQS
WapA W	MKKRK **RRNFK**	RFIAAFLVLALMISLVPADVLA KST
WprA W	MK **RRKFS**	SVVAAVLIFALIFSLFSPGT***K***AAA AGA
YceA TM	MEMFDLEFM **RRAFL**	AGGMIAVMAPILGVYLVL***RR***Q
YesM	MKKRVAGWY **RRMKI**	KDKLFVFLSLIMAVSFLFVYSGVQYA FHV
YesW	M **RRSCL**	MIRRRKRMFTAVTLLVLLVMGTSVCPV***K***A EGA
YfkN	MRIQK **RRTHV**	ENILRILLPPIMILSLILPTPPIHA EES
YkpC	MLRDLG **RRVAI**	AAILSGIILGGMSISLA NMP
YkuE	MKKMS **RRQFL**	KGMFGALAAGALTAGGGYGYA RYL
YmaC	M **RRFLL**	NVILVLAIVLFLRYVHYSL EPE
YubF TM	MQKYR **RRNTV**	AFTVLAYFTFFAGVFLFSIGLYNADNL
YuiC	MMLNMI **RRLLM**	TCLFLLAFGTTFLSVSGIEA KDL
YvhJ TM	MAERVRVRVRKKKKSK **RRKIL**	KRIMLLFALALLVVVGLGGY***K***LY
YwbN	MSDEQKKPEQIH **RRDIL**	KWGAMAGAAVAIGASGLGGLAPLVQTA AKP

[a] Amino-terminal signal peptides were identified as described in the text. Conserved residues of the RR-motif are indicated in bold letters. Only signals containing, in addition to the twin arginines, at least one other residue of the consensus sequence (R-R-X-#-#) (60) were included in this comparison. Positively charged residues in the C-domain that could function as a so-called Sec avoidance motif (23) are indicated in bold and italics. The hydrophobic H-domain is indicated in gray shading. In signal peptides with a predicted SPase I cleavage site, residues from positions −3 to −1 relative to the SPase I cleavage site are underlined, and this site is indicated with a gap in the amino acid sequence. Proteins containing additional cell wall retention signals in the mature part of the protein are indicated with a superscript W; proteins lacking a (putative) type I cleavage site, some of which contain additional transmembrane domains in their "mature" part, are indicated with a superscript TM.

age site is localized between the N- and H-domains of the prepilin signal peptide (Fig. 2). After cleavage by ComC, the hydrophobic H-domain remains attached to the mature protein. Surprisingly, potential prepilin cleavage sites are also present in two known cold shock proteins, CspB and CspC (108), and one hypothetical protein, YqaF (Table 6), which have a (predicted) cytosolic localization as they lack an H-domain or transmembrane segments. Since the catalytic domain of ComC is located at the cytoplasmic side of the membrane, it is conceivable that cytoplasmic proteins containing a ComC cleavage site are substrates for this enzyme. However, it is presently not known whether CspB, CspC, or YqaF is processed.

Signal peptides of bacteriocins and pheromones. Bacteriocins and pheromones (other than PhrA to PhrK) with a cleavable amino-terminal signal peptide form a distinct group of exported proteins that are exported via ABC transporters. Signal peptides of this class of secreted proteins cannot be predicted by the regular algorithms for signal peptide prediction, such as SignalP (see previous sections), as they consist only of N- and C-domains and completely lack a hydrophobic H-domain (Fig. 2). In *B. subtilis* 168, the three known signal peptides of this type direct the secretion of the bacteriocins sublancin 168 (203) and subtilosin (336) and the pheromone ComX (159). Thus far, no other signal peptides with a similar structure have been identified in the amino acid sequences of *B. subtilis* proteins. Obviously, this does not exclude the possibility that less related signal peptides of this type do exist, particularly in view of the fact that at least 77 (putative) ABC transporters have been identified in *B. subtilis* (149).

Protein Traffic

From the prediction of signal peptides and transmembrane regions, the percentage of the proteome that is transported from the cytoplasm to other cellular compartments can be estimated. The transport pathways followed by these (putative) preproteins will be discussed in more detail in the following paragraphs. Approximately 75% of the proteome of *B. subtilis* lacks an amino-terminal signal peptide or membrane anchor, and most of the corresponding proteins are likely to be localized in the cytoplasm. Proteins with (putative) amino-terminal signal peptides (~7%) or transmembrane segments (~18%) are likely to be targeted to the cytoplasmic membrane and (partially) translocated. A large portion (~21%) of the *B. subtilis* proteins remain linked to the membrane as transmembrane proteins (~18%), as lipid-modified proteins (~2.5%) that remain linked to the extracytoplasmic surface of the membrane by their lipid moieties, or as pilin-like structures (<0.1%). A small portion of the putative exported proteins most likely remain specifically attached to the cell wall (~0.5%; see the section on cell wall retention), whereas most of the remaining exported proteins (~4%) have the potential to pass through the cell wall and be secreted into the environment.

Most proteins seem to be exported or inserted into the cytoplasmic membrane via the Sec pathway in *B. subtilis*. Nevertheless, several alternative export pathways seem to exist. First, the recently identified twin-arginine translocation (Tat) pathway seems to be present in *B. subtilis*, as judged from the identification of signal peptides with the RR-motif and con-

TABLE 4. Predicted lipoprotein signal peptides[a]

Protein	Signal Peptide	SPase II
AppA	MKRRKTALMMLSVLMVLAIFLSA	CS
AraN	MKKMTVCFLVLMMLLTLVIAG	CS
BofC	MKRFSTAYLLLGIL	CS
CccB	MKSKLSILMIGFALSVLLAA	CG
CtaC[TM]	MVKHWRLILLLALVPLLLSG	CG
DacA	MNIKKCKQLLMSLVVLTLAVT	CL
DppE	MKRGKRMKRVKKLWGMGLALGLSFALMG	CT
FeuA	MKKISLTLLILLLALTAAA	CG
FhuD	MTHIYKKLGAAFFALLLIAALAA	CG
GerAC	MKIRILCMFICTLLLSG	CW
GerBC	MKTASKFSVMFFMLLALCG	CW
GerD	MSKAKTLLMSCFLLLSVTA	CA
GerKC	MVRKCLLAVLMLLSVIVLPG	CW
GerM	MLKKGPAVIGATCLTSALLLSG	CG
GlnH	MKKIFSLALISLFAVILLAA	CG
LplA	MKIRMRKKWMALPLAAMMIAG	CS
LytA	MKKFIALLFFILLLSG	CG
MsmE	MKHTFVLFLSLILLVLPG	CS
OppA	MKKRWSIVTLMLIFTLVLSA	CG
OpuAC	MLKKIIGIGVSAMLALSLAA	CG
OpuBC	MKRKYLKLMIGLALAATLTLSG	CS
OpuCC	MTKIKWLGAFALVFVMLLGG	CS
PbpC	MLKKCILLVFLCVGLIGLIG	CS
PrsA	MKKIAIAAITATSILALSA	CS
PstS[TM]	MKKNKLVLMLLMAAFMMIAAA	CG
QoxA[TM]	MIFLFRALKPLLVLALLTVVFVLGG	CS
RbsB	MKKAVSVILTLSLFLLTA	CS
Slp	MRYRAVFPMLIIVFALSG	CT
SpoIIIJ[TM RR]	MLLKRRIGLLLSMVGVFMLLAG	CS
SpoIVB	MPDNIRKAVGLILLVSLLSVGL	CK
XynD[TM]	MRKKCSVCLWILVLLLS	CL
YacD	MKSRTIWTIILGALLVC	CI
YbbD	MRPVFPLILSAVLFLS	CF
YbfJ	MYSTIFNIGQINKYSKLAIFMSILFL	CG
YccC	MKKQRMLVLFTALLFVFTG	CS
YcdA	MFQKKTYAVFLILLLMMFTAA	CS
YcdD	MNLPAKTFVILCILFLLDL	CF
YcdH	MFKKWSGLFVIAACFLLVAA	CG
YciB	MKLSLFIIAVLMPVILLSA	CS
YckB	MKSFMHSKAVIFSFTMAFFLILAA	CS
YckK	MKKALLALFMVVSIAALAA	CG
YclQ	MKKFALLFIALVTAVVISA	CG
YdaJ	MRHVLIAVILFFLSIGLSAG	CA
YdcC	MRKSFVLLLTGLLAVLILSA	CG
YddH	MISKKVVLPLVFSAPFIFFFVL	CI
YdeJ[RR]	MKKRRKICYCNTALLLMILLAG	CT
YdhF[RR]	MRRILSILVFAIMLAG	CS
YdhK[RR]	MSAGKSYRKKMKQRRMNMMKISKYALGILMLSLVFVLSA	CG
YdiK	MRNPVVWGMIYFAVG	CI
YerB	MKKWMTVCALCFVFFLLVS	CQ
YerH	MKKTLALAATAAVLMLSA	CS
YfiY	MKKHISMLFVFLMAVMVLSA	CN
YfjL	MKKLVFGLLAIVLFG	CG
YfmC	MRTYSNKLIAIMSVLLLA	CL
YgbA	MKKGLIVLVAVIFLLAG	CG
YhaR*	MKKVTIAAIHGAAAGLGLSLAL	CA
YhcJ	MKKWLICSFVLVLLVSFTA	CS

Continued on following page

served components of this pathway (see the section on Sec-independent protein transport). Protein secretion via this pathway was shown to be independent of Sec components in *E. coli* and plant chloroplasts. Possibly, this pathway has evolved specifically for the export of folded preproteins (65). Second, the assembly of extracellular prepilin-like structures depends on components which are, most likely, not involved in Sec-dependent protein secretion. Finally, at least three small prepeptides contain signal peptides lacking a hydrophobic domain. These prepeptides are transported across the membrane and cleaved by ABC transporters. The requirements for traveling via one of these export pathways are summarized in Fig. 4. In the follow-

TABLE 4—*Continued*

Protein	Signal Peptide	SPase II
YhcN	MFG**KK**QVLASVLLIPLLMTG	CG
YhfQ*	M**KK**TLIILTVLLLSVLTAA	CS
YjgB	M**KK**TMSAITAAAAVTS	CF
YjhA	M**KK**VLLLLFVLTIGLA**L**SA	CS
YkoI	MT**K**TI**K**TVSFAAAAILVVII	CT
YkuH ™	M**KK**LL**KK**LVVLFLSSLVIIFNVWYFII	CA
YlaJ	M**R**ILFIIIQLTLI**L**SA	CA
YmzC RR	MFESEAEL**RR**I**R**I**I**ALVWIAVFLLFGA	CG
YncB	M**KK**ILISMIAIVLSIT**L**AA	CG
YndF	M**K**S**K**L**KR**QLPAMVIVCLLMICVTG	CW
YoaJ	M**KK**IMSAFVGMVLLTIF	CF
YoaO	M**RKK**NNI**KK**WLLIIAGF**L**II	CI
YobA	MP**K**IGVSLIVLIMLIIF**L**AG	CN
YodJ	M**KK**SG**K**WFSLAAALSVTAIVGAG	CS
YojM	MH**R**LLLLMMLTALGVAG	CG
YokB	MNI**R**FSMLVCVSFIFFTGG	CA
YokF	M**KK**VLLGFAAFTLSLS**L**AA	CS
YonS	M**K**LF**KK**LGILLLITSLIL**L**AA	CK
YozF	M**KR**VLFSVIVFTAVGFTF	CQ
YphF	MG**K**L**K**CAIIFAAVVF**L**SG	CL
YpmQ	M**K**VI**K**GLTAGLIFLF**L**CA	CG
YpmR	M**K**L**R**IFSIMASLILL**L**TA	CT
YqeF	M**K**HFIILFLLLFVTAG	CE
YqgU	MLM**R**SVCFILLAVLLFS**L**SA	CK
YqiH	M**K**QTVLLLFTALF**L**SG	CS
YqiI	M**R**MLW**R**SLALCGLALT**L**AP	CA
YqiX	M**KK**WLLLLVAACITFA**L**TA	CG
YqjG ™	ML**K**TYQKLLAMGIFLIVL	CS
YrpD	MM**KK**GLLAGALTATVLFGT	CA
YrpE	MNILFS**KR**LGILTIGSLLV**L**AG	CQ
YtgA	M**R**QGLMAAVLFATFA**L**TG	CG
YtkA	M**KK**MLVVLLFSALL**L**NG	CG
YtlA	MN**R**WL**R**LGFACVGSIFLMFA**L**AA	CK
YtmJ	MN**KRK**GLVLLLSVFALLGGG	CS
YtmK	M**K**T**K**TAFMAILFSLITV**L**SA	CG
YtrF ™	MRF**K**DQVHFI**RR**NM**KK**N**R**LRVFMTILATTMA	CA
YurO	M**KK**MLLFLIIAAVSMLTIAG	CS
YusA	M**KK**LFLGALLLVFAGVMAA	CG
YutC	M**KR**TAVSLCLLTGL**L**SG	CG
YvcA	M**KK**IIFICFSLLLALTGG	CS
YvdG	MVLL**KK**GFAILAASFLAIG**L**AA	CS
YvfK	M**K**MA**KK**CSVFMLCAAVSLS**L**AA	CG
YvfO	M**K**MFFAAAIVWSA	CS
YvgL	MF**KK**YSIFIAALTAFLLVAG	CS
YvrC	M**KKR**AGIWAALLLAAVM**L**AG	CG
YwbM	MNFT**K**IAVSAGCI**L**AL	CA
YwnJ ™	MN**R**LLLAGWIFFIL**L**SV	CT
YxeA	M**KK**AMAILAVLAAAAVI	CG
YxeB	M**KK**NILLVGMLVLLLMFVSA	CS
YxeF	MVIPL**R**NKYGILFLIAV	CI
YxeM	M**K**M**KK**WTVLVVAALLAV**L**SA	CG
YxiM	M**KK**WMAAVFVMM**L**ML	CF
YxiP	M**RR**IGLCISLLVTVLVMSA	CE
YxkH	M**K**R**K**LFLSIFLLGSCLA**L**AA	CA
YybM ™	MESHIY**R**II**K**N**K**LTIIIFTIII**L**IP	CV
YycO	M**K**L**KKR**VSMFLVA**L**TM	CG
YycS	M**R**F**R**WVWLFVIMLL**L**AE	CQ

[a] Putative lipoprotein signal peptides were identified as described in the text. Positively charged lysine (K) and arginine (R) residues in the N-domain are indicated in bold letters. The hydrophobic H-domain is indicated in gray shading. The residues at positions −3 to +1, forming the lipobox, are underlined. The SPase II cleavage site is indicated with a gap in the amino acid sequence. Leucine residues at position −3 and strictly conserved cysteine residues at position +1 are indicated in bold letters. Lipoproteins containing (putative) transmembrane domains in the mature part of the protein are indicated with a superscript TM. Conserved residues of the RR-motif (R-R-X-#-#) (60) in the N-domains of the putative lipoproteins SpoIIIJ, YdeJ, YdhF, YdhK, and YmzC (indicated with a superscript RR) are enlarged. Based on theoretical considerations, the putative start sites of the potential lipoproteins YhaR and YhfQ (indicated with ∗) have been modified in a recent update of SubtiList. If the new annotation is correct, it is uncertain whether YhaR and YhfQ are lipoproteins.

TABLE 5. Amino acid residues around (putative) SPase II cleavage sites[a]

Position and residue	Frequency (% of total)
−3	
L	65
V	8
F	7
T	6
I	5
M	2
G	2
S	<1
W	<1
−2	
A	36
S	24
T	11
I	6
V	6
G	6
M	2
L	2
C	2
P	2
F	2
L	2
D	<1
N	<1
−1	
A	39
G	35
L	8
I	4
S	4
V	2
T	2
F	2
P	2
M	<1
C	<1
E	<1
+1	
C	100
+2	
G	30
S	28
A	9
I	4
T	4
F	4
W	4
K	4
L	4

[a] The frequency of a particular amino acid at each position is given as the percentage of the total number of predicted lipoprotein signal peptides in which it appears.

ing sections, each of these export pathways will be discussed in more detail.

THE Sec-DEPENDENT SECRETION MACHINERY

The various components of the Sec-dependent secretion machinery can be divided into six groups: cytosolic chaperones, the translocation motor (SecA), components of the translocation channel (SecYEG and SecDF-YajC), SPases, SPPases, and, finally, folding factors that function at the *trans* side of the membrane. The main components of the secretion machinery of *B. subtilis* are depicted in Fig. 5 and listed in Table 7. In addition, the homologous and/or analogous components from *E. coli*, the archaeon *Methanococcus jannaschii*, and the eukaryon *S. cerevisiae* are listed in Table 7. As protein secretion has not been studied experimentally in archaea, the identification of components of the secretion machinery of *M. jannaschii* is based entirely upon data deduced from the genome sequence (44).

Cytosolic Chaperones

Most proteins that are destined for export can only be translocated across the membrane in a more or less unfolded conformation that allows them to pass through the translocation channel of the Sec pathway. To facilitate this, cytosolic factors aid in maintaining these preproteins in a so-called translocation-competent state. Such factors, called chaperones, bind to preproteins and prevent their folding and aggregation. Some of these chaperones are secretion dedicated and assist in protein targeting to the translocase.

Secretion-dedicated chaperones. In *B. subtilis*, the only secretion-specific chaperone thus far identified is the Ffh protein (fifty-four homologue), a GTPase that is homologous to the 54-kDa subunit of the eukaryotic signal recognition particle (Srp54) (122). This protein forms a complex (denoted SRP) with the small cytoplasmic RNA (scRNA) that is functionally related to the eukaryotic 7S RNA (called scR1 RNA in *S. cerevisiae*) and the *E. coli* 4.5S RNA (186, 187). Recent data have shown that HBsu, a histone-like protein of *B. subtilis*, is also associated with the scRNA. Notably, HBsu was shown to bind to a region of scRNA that is not conserved in the 4.5S RNA of *E. coli*, suggesting that the *E. coli* SRP lacks an HBsu-like component (188, 333). The ternary ribonucleoprotein SRP complex of *B. subtilis* binds to the signal peptides of nascent chains emerging from the ribosome and is targeted to the membrane with the aid of the FtsY protein (also called Srb) (200). FtsY is a homologue of the eukaryotic SRP receptor α-subunit (DPα) that is essential for SRP-dependent protein secretion and cell viability, like the Ffh protein. In eukaryotic cells, SRP-dependent protein translocation occurs cotranslationally. The SRP of *S. cerevisiae* consists of a complex of seven subunits and the 7S RNA. Two of these subunits, Srp7 (Srp9 in mammalian cells) and Srp14, are responsible for a translation arrest as soon as the signal peptide emerges from the ribosome (262). The whole complex, consisting of the ribosome, nascent chain, and SRP, docks onto the SRP receptor (also termed docking protein), which consists of DPα and DPβ. Next, SRP is released and polypeptide translation by the ribosome is resumed. Protein synthesis is likely to provide the driving force for cotranslational protein translocation across membranes (249). A similar SRP-mediated translation arrest probably does not occur in eubacteria. First, it was shown that *E. coli* SRP and FtsY do not arrest translation in a eukaryotic in vitro translocation assay (222). Second, eubacteria lack the SRP components that are responsible for translation arrest in eukaryotes. Moreover, a specific translation arrest may not even be required for cotranslational translocation in eubacteria, because the traffic distances are short and the protein translocation rates are high compared to the translation rate (for *E. coli*, estimated at approximately 10-fold) (225, 302).

In *E. coli*, a second protein-targeting pathway utilizes the SecB protein that was shown to bind to the mature regions of a subset of preproteins (148) and the carboxyl terminus of SecA (92). Strikingly, recent results showed that the SecB-binding motif, consisting of a stretch of ~9 amino acids enriched in aromatic and basic residues, occurs in SecB-dependent and -independent secretory proteins and in cytosolic proteins (141). Moreover, SecB appeared to bind these three

TABLE 6. Predicted type IV prepilin signal peptides and related sequences[a]

Protein	ComC	Signal Pepide
ComGC	MNE**KG**	**F**TLV**E**MLIVLFIISILLLITIPNVTKHNQTI
ComGE	MWREN**KG**	**F**STI**E**TMSALSLWLFVLLTVVPLWDKLMAD
ComGD	MNIKLNEE**KG**	**F**TLL**E**SLLVLSLASILLVAVFTTLPPAYDNTAVR
ComGG	MYRT**RG**	**F**IYPAVLFVSALVLLIVNFVAAQYISRCMFEK
CspC	MEQGTVKWFNAE**KG**	**F**GFI**E**RENGDDVFVHFSAIQSDGFKSLDEGQKVS
CspB	MLEGKVKWFNSE**KG**	**F**GFI**E**VEGQDDVFVHFSAIQGEGFKTLEEGQAVS
YqaF	MMRKWLKKNRLE**KG**	**F**TQE**E**VAKAAQIGRAYYTMIENGTRKPSVIVSKK

[a] Prepilin-like signal peptides were identified as described in the text. The hydrophobic H-domain is indicated in gray shading. The ComC recognition sequence in prepilin signal peptides and putative ComC recognition sequences in CspC, CspB, and YqaF are indicated in bold letters. Predicted ComC cleavage sites are indicated with a gap in the amino acid sequence.

classes of proteins with more or less similar affinities. This suggests that SecB may also be regarded as a general chaperone that promotes protein translocation by its specific binding to SecA, together with an associated preprotein (141). For *E. coli*, it was shown that the SRP route converges with the SecB-dependent targeting route at the translocase (302), and it was proposed that the SRP route acts primarily cotranslationally, while the SecB-dependent route acts mainly posttranslationally. Recent studies showed that SecA, SecB, SecE, and ATP are dispensable for the transfer of certain (nascent) membrane proteins to SecY and their subsequent insertion into the membrane, suggesting that SRP-FtsY and SecB-SecA constitute distinct targeting, or even translocation, routes (59, 69, 142, 256). The choice between these two routes seems to depend largely on the hydrophobicity of the targeting signal, as the more hydrophobic signal peptides target proteins primarily into the SRP pathway (300, 301). Altogether, these findings suggest that the SRP route of *E. coli* is mainly involved in the targeting of inner membrane proteins, whereas the SecA-SecB route is primarily involved in the targeting of periplasmic and outer membrane proteins (15, 256, 257, 299). As the signal peptides of *B. subtilis* are, on average, longer and more hydrophobic than those of *E. coli* (321), thereby closely resembling transmembrane segments of integral membrane proteins (see above), it seems likely that the majority of

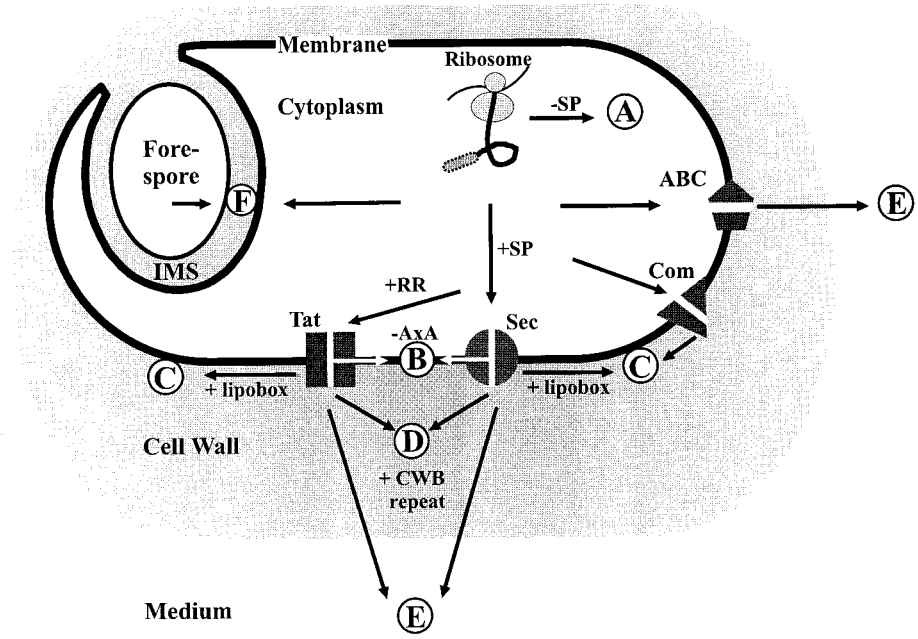

FIG. 4. Predicted protein transport pathways in *B. subtilis*. Based on the predictions of signal peptides and various retention signals, it is hypothesized that at least four different protein transport pathways exist in *B. subtilis* that can direct proteins to at least five different subcellular destinations. Ribosomally synthesized proteins can be sorted to various destinations depending on the presence (+SP) or absence (−SP) of an amino-terminal signal peptide and specific retention signals such as lipid modification or cell wall-binding repeats (CWB). (A) Proteins devoid of a signal peptide remain in the cytoplasm. (B) Proteins with one or more membrane-spanning domains are inserted into the membrane either spontaneously (not shown), via the Sec pathway or, according to our predictions (Table 3), via the Tat pathway (+RR). (C) Proteins which have to be active at the extracytoplasmic side of the membrane can either be lipid-modified proteins (+lipobox) exported via the Sec or Tat pathways or prepilins exported by the Com system. (D) Proteins that need to be retained in the cell wall can be exported via the Sec or Tat pathway. In order to be retained, the mature part of these proteins contains cell wall-binding repeats (+CWB). (E) Proteins can be secreted into the medium via the Sec or Tat pathway or by ABC transporters. (F) Different mechanisms can be employed to transport proteins to the IMS of endospores.

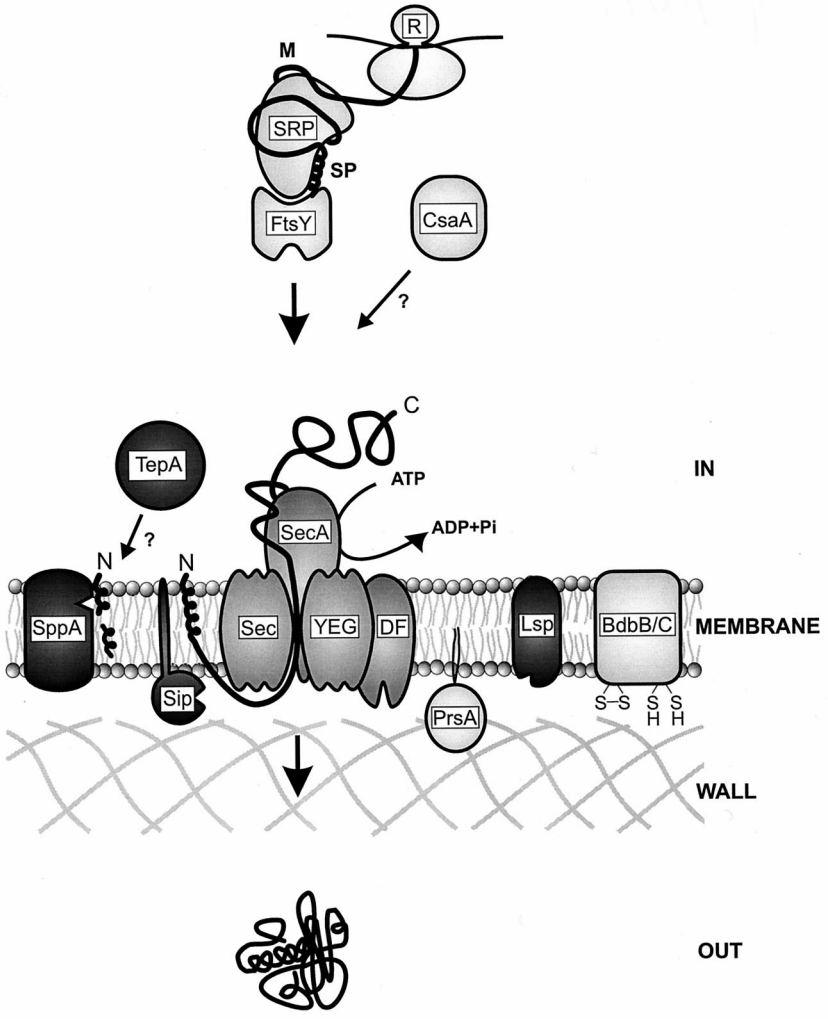

FIG. 5. Main components of the Sec-dependent *B. subtilis* protein secretion machinery. The SRP complex consists of Ffh and the scRNA. See text for further details. C, carboxyl terminus; M, mature protein; N, amino terminus; R, ribosome; SP, signal peptide.

B. subtilis proteins are secreted in an SRP-dependent manner. This view seems to be supported by recent experiments of Hirose et al. (119), showing that the secretion of a large number of *B. subtilis* proteins was directly or indirectly Ffh dependent. Notably, the secretion of most of these proteins depended on SecA as well, suggesting that SRP-dependent protein secretion does not bypass SecA in *B. subtilis*.

Interestingly, no SecB homologue is present in *B. subtilis*, but it is conceivable that a SecB analogue does exist in this organism. For example, the SecB-binding domain of *E. coli* SecA is highly conserved in the SecA protein of *B. subtilis* (92). This binding domain might serve as a docking site for a SecB analogue, but obviously, other sites in the *B. subtilis* SecA protein could also serve this purpose. A candidate for a SecB analogue in *B. subtilis* is the CsaA protein, which was identified as a suppressor of an *E. coli* SecA(Ts) mutant (178). Recently, it was demonstrated that CsaA has chaperone-like activities (179) and affinity for SecA and preproteins (179a). These findings suggest that CsaA has an export-related function in *B. subtilis*. In conclusion, it is very well conceivable that *B. subtilis*, like *E. coli*, does contain two distinct routes for protein targeting to the translocase: first, a cotranslational targeting route via SRP, and second, a posttranslational targeting route in which CsaA might participate (Fig. 6). It has to be noted, however, that CsaA does not seem to bind to the conserved SecB-binding domain in SecA (179a). Thus, the precise role of CsaA in protein secretion in *B. subtilis* remains to be elucidated.

General chaperones. In addition to secretion-dedicated chaperones, chaperones with a general function in protein unfolding and folding might also function in protein translocation. Lill et al. (154) were the first to demonstrate that the so-called trigger factor (TF) is a cytosolic ribosome-bound protein that can maintain the translocation competence of the precursor form of the outer membrane protein OmpA in vitro. However, later studies indicated that depletion of TF did not block the export of proOmpA (109). Recently, it was shown that TF is a ribosome-bound peptidyl-prolyl *cis/trans* isomerase (PPIase, FK506 binding protein type [FKBP]) that interacts with both secretory and cytosolic proteins (117, 300, 301). Thus, the interaction with TF might represent a decision point for proteins to enter either the GroEL/ES folding pathway when these proteins are to remain cytosolic or to enter a targeting pathway to the translocase for secretory proteins. In addition, recent results indicate that secretory proteins are directed into the SecB-SecA-mediated posttranslational targeting pathway by means of their preferential recognition by TF (15). In *B. subtilis*, it was shown that TF, together with a second cytoplasmic PPIase (cyclophilin, also called PpiB), ac-

TABLE 7. Components of Sec-dependent protein export machineries of *B. subtilis*, *E. coli*, *M. jannaschii*, and the ER of *S. cerevisiae*[a]

Component	*B. subtilis*	*E. coli*	*M. jannaschii*	*S. cerevisiae*
Secretion-dedicated chaperones	Ffh, FtsY, scRNA	Ffh, FtsY, 4.5S RNA	Srp54/19, FtsY, 7S RNA	SRP complex,[b] DPα/β
		SecB		
	CsaA (?)	YgjH (?)		
General chaperones	GroEL, GroES	GroEL, GroES		
	DnaK, DnaJ, GrpE	DnaK, DnaJ, GrpE		Hsp70, Ydj1
	Trigger factor	Trigger factor		
Translocation motor	SecA	SecA		
				Ribosome
				Kar2 (Bip[c])
Translocation channel	SecY	SecY	SecY	Sec61, Ssh1 (Sec61α[c])
	SecG	SecG	Sec61β	Sbh1, Sbh2 (Sec61β[c])
	SecE	SecE	SecE	Sss1 (Sec61γ[c])
	SecDF, YrbF	SecD/F, YajC	SecD/F	
				Sec62/63
				Sec66/67
SPases	SipS/T/U/V/P	LepB		
	SipW		Sec11	Sec11
	LspA	LspA		
SPPases	SppA	SppA	SppA	
	TepA			
		OpdA		
Foldases (*trans*-acting)	PrsA	SurA, PpiD, RotA, FkpA		CPR2, CPR4, CPR5, CPR8, FPR2
	BdbA/B/C	DsbA/B/C/D/E/G		PDI, Ero1, Eug1
				Kar2 (BiP[c]), Lhs1 (Hsp70[c])

[a] Proteins with similar functions are placed in the same horizontal row.
[b] The SRP complex consists of scR1 RNA, Srp72p, Srp68p, Srp54p, Sec65p, Srp21p, Srp14p, and Srp7p (338).
[c] Synonymous names for mammalian homologues.

counts for the entire PPIase activity in the cytoplasm (107). A direct involvement of TF or cyclophilin in protein secretion by *B. subtilis* has not been reported. However, it was recently shown that TF of the gram-positive eubacterium *Streptococcus pyogenes* is important for the secretion of the cysteine proteinase SCP (158). Interestingly, TF was required both for guiding SCP into the secretory pathway and for establishing an active conformation after translocation. This suggests that the *cis-trans* isomerization of certain peptidyl-prolyl bonds before translocation is important for the folding of the protein after translocation. However, an alternative explanation is that TF is involved in the secretion of an extracellular foldase that is required for folding of SCP at the *trans* side of the membrane.

In *E. coli*, it was shown that GroEL and GroES are important for the translocation of the SecB-independent precursor β-lactamase (21, 150). Furthermore, it was shown that GroEL interacts with SecA, and hence it was suggested that GroEL might be involved in the release of SecA from the membrane (22). Also, DnaK, DnaJ, and GrpE were shown to be involved in the export of a number of SecB-independent proteins, such as alkaline phosphatase, β-lactamase, and ribose-binding protein (327, 328). Similarly, DnaK and DnaJ homologues in *S. cerevisiae*, called Hsp70 and Ydj1, respectively, were shown to be involved in the posttranslational translocation of proteins across the ER membrane (338). In contrast, a role in protein secretion could not thus far be demonstrated for the corresponding heat shock chaperones in *B. subtilis* (T. Wiegert and W. Schumann, personal communication). Nevertheless, secretion of an antidigoxin single-chain antibody, which has the tendency to accumulate in inclusion bodies, was shown to be improved by about 60% through concerted overproduction of the GroEL-ES and DnaK-DnaJ-GrpE chaperone machineries (331). Even though the latter observation suggests that the effects of overproduction of these chaperone machineries are caused by the prevention of aggregation, the possibility that GroEL-ES and/or DnaK-DnaJ-GrpE are more directly involved in protein secretion by *B. subtilis* cannot presently be excluded.

The Translocase

The preprotein translocation machinery of the *E. coli* Sec pathway consists of at least seven proteins: SecA, which is the translocation motor, and the integral membrane proteins SecD, SecE, SecF, SecG, SecY, and YajC. Homologues of all of these components have been identified in *B. subtilis*. In the current model of preprotein translocation, which is based largely on results obtained in *E. coli*, several successive steps in the translocation of proteins are proposed (77, 79, 83, 304). First, SecA binds to acidic phospholipids and SecY (82, 110, 156, 161, 270) and is activated for recognition of SecB and the preprotein (110). Preprotein binding is followed by the binding and hydrolysis of ATP (155). The binding of ATP causes major conformational changes of SecA (72, 303), leading to a release of SecB (92, 93) and insertion of the carboxyl terminus of SecA into the membrane (85, 86, 94, 224). This membrane insertion, which occurs through the translocase complex (85, 87, 88, 224) promotes the translocation of a short fragment of the preprotein (251). Next, ATP is hydrolyzed by SecA, leading to release of the preprotein and deinsertion of SecA (85, 251). Once protein translocation is initiated by SecA, further translocation is driven by both repeated cycling of SecA through ATP binding and hydrolysis and the proton motive force (78, 100, 261).

The *B. subtilis* gene encoding SecA was initially identified as a gene called *div*, mutations in which affected cell division, sporulation, germination, protein secretion, autolysis, and the development of competence for DNA binding and uptake (236, 237). In fact, cloning and sequencing of the *div* gene, which is essential for cell viability, revealed that it encodes

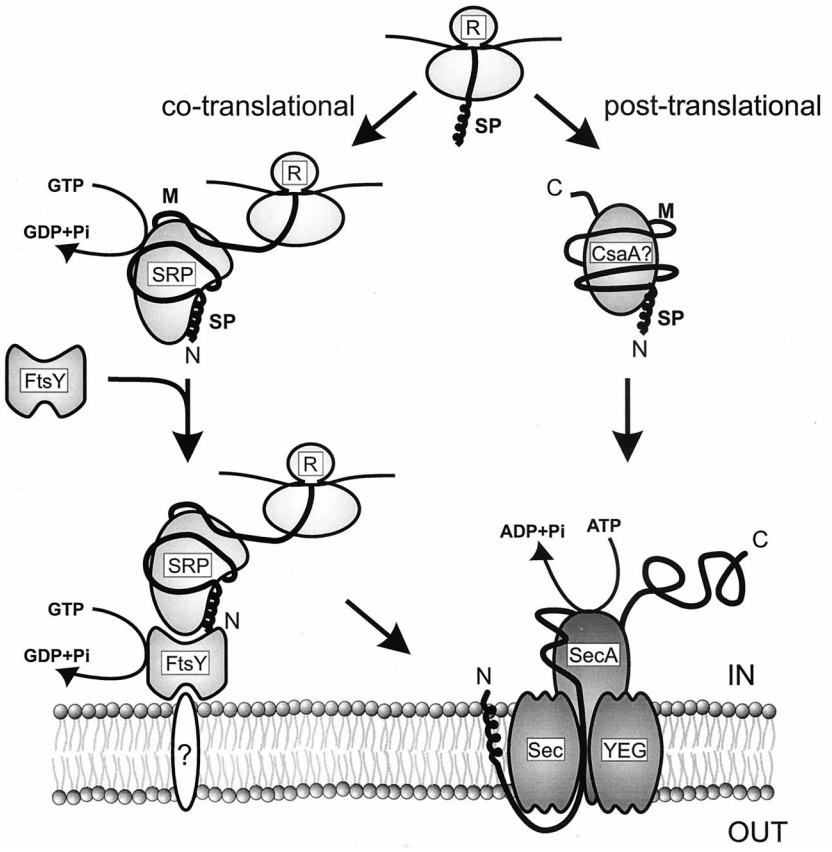

FIG. 6. Model for protein targeting to the *B. subtilis* Sec translocase. Precursor proteins with the most hydrophobic targeting signals are bound cotranslationally by the SRP complex. The complex formed by the nascent chain, ribosome, and SRP docks the preprotein at an unidentified site at the membrane with the aid of FtsY. The subsequent release of the nascent chain and ribosome from the SRP-FtsY complex appears to be preceded or accompanied by GTP binding to the Ffh protein in SRP and FtsY. Hydrolysis of GTP bound to both Ffh and FtsY mediates the dissociation and recycling of these targeting components. A subset of precursor proteins containing relatively fewer hydrophobic targeting signals interact posttranslationally with an unidentified chaperone, possibly CsaA, which targets the complex to the translocase. After their binding to the Sec translocase, precursors are translocated across the membrane through cycles of ATP-dependent insertion and deinsertion of SecA into the translocation channel (see text for details). This model is largely based upon the model for the *E. coli* targeting routes proposed by Valent et al. (302). C, carboxyl terminus; M, mature protein; N, amino terminus; R, ribosome; SP, signal peptide.

SecA (238). In addition, the *B. subtilis secA* gene was cloned independently, using hybridization with an *E. coli secA* probe (201). *B. subtilis* SecA can complement *E. coli* SecA mutants, provided that the protein is expressed at moderate levels (140). Notably, yeasts and archaea do not contain SecA homologues even though they contain a Sec-type protein-conducting channel (see below). In *S. cerevisiae*, the driving force for protein translocation across the ER membrane is generated either by the ribosome, in the case of cotranslational translocation, or by the ER-luminal Kar2 protein (an Hsp70 homologue), in the case of posttranslational translocation (249). The fact that SecA is absent from archaea, at least the ones for which the genome has been sequenced completely, suggests that these organisms use either another cytoplasmic ATPase, the SRP pathway, and/or protein synthesis as a force generator for protein translocation.

Notably, in addition to its role in preprotein translocation, SecA of *B. subtilis* could also fulfill the role of an export-specific chaperone, as suggested by Herbort et al. (116). Such a role for SecA of *B. subtilis* would be consistent with its recently documented low affinity for SecYEG (283). The latter observation implies that, at least in *B. subtilis*, the levels of cytosolic SecA are relatively high, which would facilitate early interactions with proteins destined for export.

A heterotrimeric complex of SecY, SecE, and SecG forms the main core of the translocation channel (42). This complex is found not only in eubacteria but also in eukaryotes (Sec61p complex) (106) and archaea (216). The SecY and SecE homologues in eukaryotes are called Sec61α and Sec61γ, respectively. SecG is not conserved, but this protein could be a functional analogue of the eukaryotic Sec61β. Based on sequence similarity and the observation that archaea probably contain a Sec61β homologue rather than SecG, it was suggested that the archael translocase is more related to the eukaryotic Sec61p complex (216). During the last decade, homologues of SecY, SecE, and SecG have been identified in *B. subtilis*, either by complementation studies using *E. coli sec* mutants or by DNA sequencing (131, 185, 280, 312). SecY, SecE, and SecG of *B. subtilis* are membrane proteins, with 10, 1, and 2 membrane-spanning domains, respectively (131, 185, 312). Notably, *B. subtilis* SecE is considerably smaller than *E. coli* SecE and has only one membrane-spanning domain, while *E. coli* SecE has three such domains (250). Nevertheless, SecE of *B. subtilis* was able to complement the cold-sensitive and export-defective phenotype of an *E. coli* SecE mutant, showing that it is a true SecE homologue (131). Based on data from posttranslational protein transport experiments in the *S. cerevisiae* ER membrane, Plath et al. (213) postulated that SecE and signal peptides bind to the same or overlapping regions in SecY and that SecE functions as a surrogate signal peptide when the SecY channel is in its closed form in the absence of translocating protein. Upon the arrival of a signal peptide, it would displace SecE and thus open the SecY channel for transport. In contrast

to SecY and SecE, SecG is not strictly required for preprotein translocation and cell viability. Nevertheless, it is required for efficient translocation, possibly by facilitating the movement of preproteins through the translocation channel in concert with the insertion and deinsertion cycles of SecA (165). The absence of SecG from *E. coli* causes a cold-sensitive growth phenotype, as frequently encountered in *E. coli* strains in which protein secretion via the Sec pathway is compromised (215). Similarly, disruption of the *B. subtilis secG* (*yvaL*) gene caused secretion defects that resulted in cold-sensitive growth (312), confirming the earlier conclusion by Bolhuis et al. (27) that protein translocation in *B. subtilis* is intrinsically cold sensitive, as it is in *E. coli*. Furthermore, the cold sensitivity of the *B. subtilis secG* mutant was exacerbated by overproduction of secretory preproteins. Interestingly, the growth and secretion defects of the *B. subtilis secG* mutant could be complemented by the expression of the *E. coli secG* gene even when secretory preproteins were overproduced. Finally, consistent with the role of SecG in *E. coli*, *B. subtilis* SecG stimulated the ATP-dependent in vitro translocation of the precursor pre-PhoB by the *B. subtilis* SecA-SecYE complex (283).

In addition to the genes encoding the SecYEG core elements of the translocase, a gene encoding the SecDF protein was also identified in *B. subtilis* (27). In contrast to the *secD* and *secF* genes identified in most other organisms, *B. subtilis* was shown to contain a natural gene fusion between the equivalents of *secD* and *secF*. Consequently, SecDF of *B. subtilis* is a molecular Siamese twin, with 12 putative transmembrane domains. Notably, SecDF shows both sequence similarity and structural similarity to secondary solute transporters. It was demonstrated that *B. subtilis* SecDF, which is not essential for cell viability, is merely required to maintain a high capacity for protein secretion (27). Unlike the SecD subunit of *E. coli* (166), the *B. subtilis* SecDF protein does not seem to be required for the release of a mature secretory protein from the membrane. It has been suggested that SecD and SecF of *E. coli* modulate the cycling of SecA (83, 86, 137). However, it was also noted that archaea, which contain separate SecD and SecF proteins, do not contain a SecA homologue (216). Therefore, it is conceivable that SecD-SecF has another function in protein translocation, such as assembly of the translocase (216) or clearing of the translocation channel from signal peptides or misfolded proteins (27). The latter idea would be consistent with the observation that SecDF shows structural similarity to secondary solute transporters.

For *E. coli*, it was shown that SecD and SecF form a heterotrimeric subcomplex with a third protein (denoted YajC) and that this complex constitutes a large "holoenzyme" with the SecYEG complex (83). YajC is specified by the first gene of the SecDF operon. A gene encoding a homologue of the *E. coli* YajC protein, denoted *yrbF*, was also identified on the *B. subtilis* genome (53% identical plus conservative residues), but its involvement in protein secretion has not been documented so far. This *yrbF* gene is located in the same chromosomal region as the *secDF* gene but, in contrast to *E. coli*, it is not cotranscribed with *secDF* (27). Disruption of the *yajC* gene of *E. coli* did not have a clear effect on protein export, but it was shown that overproduction of YajC suppresses the dominant-negative phenotype of the *secY-d1* mutation, an internal in-frame deletion in the *secY* gene (287).

Finally, the Sec translocon in the ER of eukaryotic cells contains, in addition to the Sec61 core components, a number of other membrane proteins (338). These include the components of the Sec62/63 and Sec66/67 (also called Sec71/72) complexes. In addition, translocation complexes in the ER of mammalian cells contain the TRAM protein. The function of these proteins is not fully clear, but they may have functions analogous to those proposed for the SecD/F proteins (216).

Type I SPases

SPases remove signal peptides from secretory preproteins when their C-domain emerges at the extracytoplasmatic side of the membrane. This reaction is a prerequisite for the release of the mature secretory protein from the membrane (64, 67). One of the most remarkable features of the *B. subtilis* protein secretion machinery is the presence of multiple, paralogous type I SPases. This in contrast to the situation in many eubacteria, archaea, and yeasts, in which one type I SPase seems to be sufficient for the processing of secretory preproteins (44, 67, 105, 269). For most eukaryotic species, however, the presence of two paralogous SPases appears to be characteristic (67). The largest numbers of known paralogous SPases appear to be present in the archaeon *Archaeoglobus fulgidus*, which contains four genes for type I SPases (139), and *B. subtilis*, in which seven *sip* genes for type I SPases have been identified so far. Five of the *sip* genes of *B. subtilis* (*sipS*, *sipT*, *sipU*, *sipV*, and *sipW*) are located on the chromosome (26, 289, 290, 307, 308); two additional *sip* genes (*sipP*) are located on plasmids that were identified in *natto*-producing strains of *B. subtilis* (171, 172). As was shown for *E. coli* (66, 306) and *S. cerevisiae* (25), SPase I activity in *B. subtilis* is essential for cell viability. Although all five chromosomally encoded SPases can process secretory preproteins, only SipS and SipT are of major importance for preprotein processing and viability, whereas SipU, SipV, and SipW have a minor role in protein secretion (290). Notably, SipS and SipT can be functionally replaced by the plasmid-encoded SPase SipP (292). The latter three SPases are therefore considered to be the "major" SPases, which have substrate specificities that differ at least partly from those of SipV, SipU, and SipW, the "minor" SPases (Fig. 7). These findings indicate that the minor SPases are specifically required for the processing of a subset of the 180 predicted secretory preproteins. Indeed, SipW seems to be specifically involved in the processing of pre-TasA and pre-YqxM, two preproteins that are encoded by genes flanking the *sipW* gene (276, 277, 278). Surprisingly, SipW shows high degrees of sequence similarity not only to certain SPases found in sporulating gram-positive eubacteria, but also to the SPases of archaea and the eukaryotic ER membrane. Together these SipW-like SPases form the subfamily of ER-type SPases. In contrast, all other known *B. subtilis* SPases are of the prokaryotic type (P-type). Such P-type SPases have thus far been found exclusively in eubacteria, mitochondria, and chloroplasts (290). As demonstrated by site-directed mutagenesis of various P-type SPases, including SipS of *B. subtilis* (298, 305), and by X-ray crystallography of the *E. coli* SPase I (202), the P-type SPases make use of a serine-lysine catalytic dyad. In all known eubacterial P-type SPases, the active-site serine residue is predicted to be localized at the extracytoplasmic membrane surface. In SipS, SipT, SipU, SipV, and SipP of *B. subtilis*, this serine residue is kept in position by a unique amino-terminal membrane anchor. In contrast to the latter SPases, SipW appears to have a carboxyl-terminal membrane anchor in addition to an amino-terminal membrane anchor that precedes its active-site serine residue (290). However, the major difference between P- and ER-type SPases is that the catalytic lysine residue of the P-type SPases is replaced with a histidine residue in the ER-type SPases (67, 290, 307). Recent studies have shown that conserved serine, histidine, and aspartic acid residues are critical for the activity of SipW and the ER SPase Sec11 of *S. cerevisiae*, indicating that

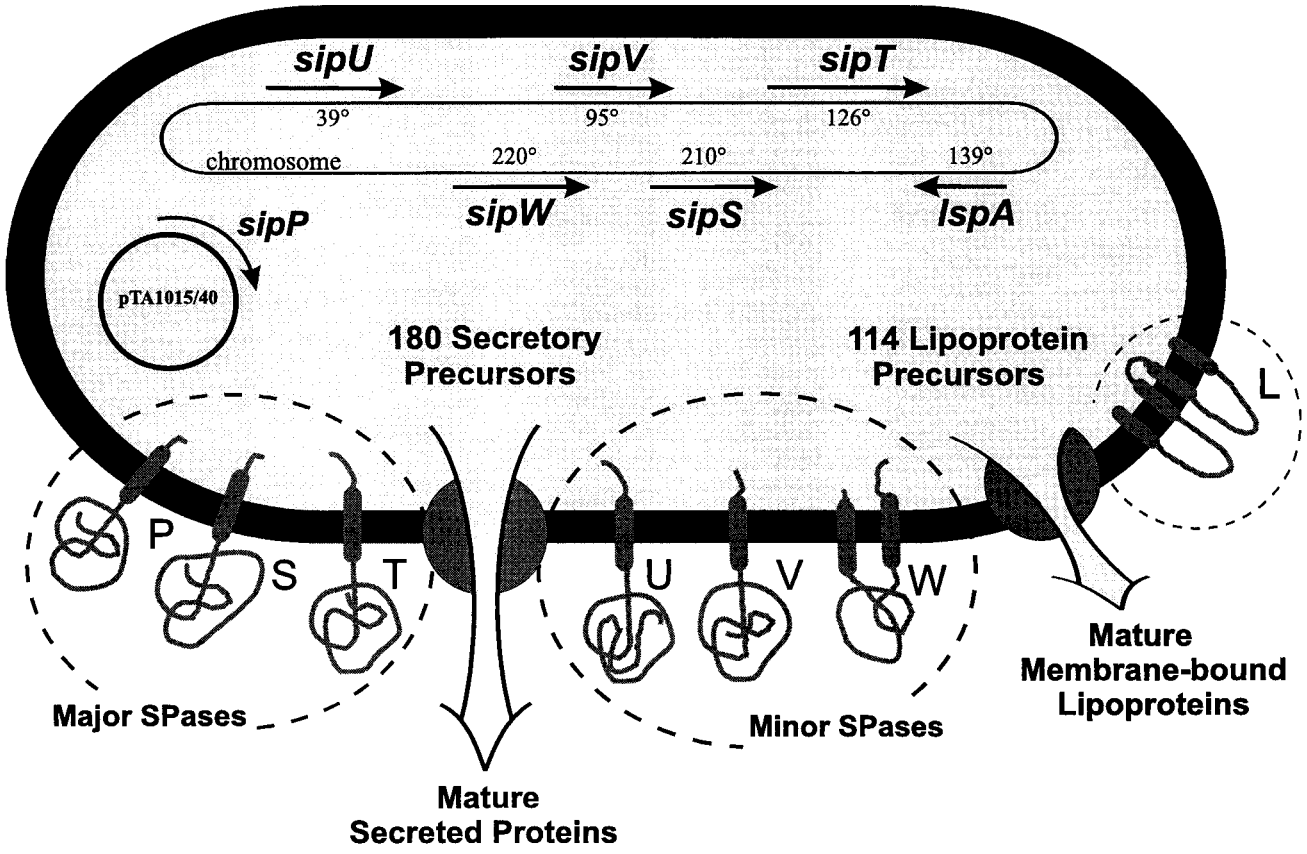

FIG. 7. Type I and II SPases of *B. subtilis*. The type I SPases, responsible for the processing of the 180 predicted secretory preproteins, can be divided into two groups: the major SPases SipS (S), SipT (T), and SipP (P), which are important for cell viability, and the minor SPases SipU (U), SipV (V), and SipW (W), which are not important for cell viability under laboratory conditions (290, 292). SipP is encoded by plasmid-borne genes on the plasmids pTA1015 and pTA1040, which are present in certain *natto*-producing *B. subtilis* strains. All other SPases are chromosomally encoded. The transcription of the genes for the major SPases increases during the postexponential growth phase in concert with the genes for most secretory proteins, whereas the minor SPases are transcribed at a low level during all growth phases. SipW is the only ER-type SPase, showing a high degree of similarity to eukaryotic and archaeal SPases. In contrast to the prokaryotic (P-)type SPases of *B. subtilis*, which have one amino-terminal membrane anchor, SipW appears to have an additional carboxyl-terminal membrane anchor. Finally, *B. subtilis* contains only one gene (*lsp*) for a type II SPase (L) with four membrane anchors, which is required for the processing of the 114 predicted lipoproteins (291) (see text for details).

ER-type SPases employ a Ser-His-Asp catalytic triad or, alternatively, a Ser-His catalytic dyad (293a, 311).

Even though it is well established that various type I SPases of *B. subtilis* have different substrate specificities, the molecular basis for these differences with respect to the composition of the C-domain of signal peptides and the structure of the active sites of the different SPases is presently unknown.

Lipoprotein Processing by SPase II

In contrast to the type I SPases, *B. subtilis* contains only one gene for a type II SPase (*lsp*) (223), which is specifically required for the processing of lipid-modified preproteins. All known SPases of this type are integral membrane proteins with four (putative) membrane-spanning segments, the amino and carboxyl termini having a predicted cytosolic localization (183, 223, 293). The potential active site of SPase II, formed by two aspartic acid residues, is located in close proximity to the extracytoplasmic surface of the membrane, similar to the active-site serine residue of type I SPases (293).

Interestingly, cells lacking SPase II are viable under standard laboratory conditions. This indicates that processing of lipoproteins by SPase II in *B. subtilis* is not strictly required for lipoprotein function, as at least one lipoprotein, PrsA, is essential for viability (144, 291). Although certain lipoproteins are required for the development of genetic competence, sporulation, and germination, these developmental processes were not detectably affected in the absence of SPase II. Cells lacking SPase II accumulated lipid-modified precursor and, surprisingly, mature-like forms of the lipoprotein PrsA, which is involved in the folding of secreted proteins. These forms of PrsA appeared to be reduced in activity, as the secretion of the *B. amyloliquefaciens* α-amylase AmyQ, the folding of which is dependent on PrsA, was strongly impaired (291). It is presently not clear which proteases are responsible for the alternative processing of PrsA in the absence of SPase II. However, the involvement of type I SPases in this process appears to be highly unlikely (291). The cellular level of another lipoprotein, CtaC, is strongly reduced in the absence of SPase II, indicating that lipoprotein processing is important for the stability of certain proteins (17).

Diacylglyceryl modification of the cysteine residue at position +1 of the mature lipoprotein in lipoprotein precursors is catalyzed by the lipoprotein diacylglyceryl transferase (Lgt). This lipid modification is a prerequisite for processing of the lipoprotein precursor by SPase II (103, 147, 242, 243, 244, 294). Similar to the disruption of the *lsp* gene for SPase II, disruption of the *lgt* gene results in the accumulation of unprocessed (and unmodified) lipoproteins without affecting growth and cell viability (153, 291). Like SPase II, Lgt is required for the stability of several lipoproteins and the efficient secretion of AmyQ (17, 153). Interestingly, the processing rate of secreted nonlipoproteins was retarded in the absence of SPase II, which must be

attributed to the malfunction of lipoproteins other than PrsA (291). Two candidate proteins that might be responsible for this effect are the putative lipoproteins SpoIIIJ and YqjG (Table 3), which show significant sequence similarity to the mitochondrial Oxa1 protein. The latter protein was shown to be required for export of the amino and carboxyl termini of the mitochondrially encoded precursor of cytochrome *c* oxidase subunit II (pre-CoxII) from the mitochondrial matrix to the intermembrane space (114, 115), proteolytic processing of pre-CoxII (13), and assembly of the cytochrome *c* oxidase and oligomycin-sensitive ATP synthase complexes (3, 33). As mitochondria lack Sec components, Oxa1p seems to represent a component of a specific protein export and/or assembly machinery that might be conserved in eukaryotic organelles and eubacteria (115, 235). Consistent with this hypothesis, it was recently shown that the *E. coli* homologue of SpoIIIJ/YqjG and Oxa1p, denoted YidC, is associated with the Sec translocase (256a).

Finally, processed lipoproteins of *E. coli* are further modified by aminoacylation of the diacylglyceryl-cysteine amino group (103, 147, 242, 243, 244, 294). The latter lipid modification step does not seem to be conserved in all eubacteria, as many organisms, including *B. subtilis*, lack an *lnt* gene for the lipoprotein *N*-acyltransferase (291).

SPPases

After being cleaved off from the mature protein, signal peptides are rapidly degraded. In *E. coli*, the signal peptide of the major lipoprotein (Lpp; also called Braun's lipoprotein) was shown to be degraded by the membrane-bound protease IV (also called signal peptide peptidase, encoded by the *sppA* gene) (123, 125, 126). Nevertheless, in the absence of protease IV, significant levels of signal peptide degradation were observed (282), suggesting that other proteases can replace protease IV in this process. In addition to the protease IV, the cytoplasmic oligopeptidase A (OpdA) was shown to be involved in the degradation of the Lpp signal peptide (197, 198). Current models imply that protease IV cleaves the signal peptide in the membrane in two fragments (Fig. 1), which are further degraded in the cytoplasm by OpdA (198). Interestingly, independent of its effect on natural signal peptides, OpdA was also identified in suppressor screens using secretory proteins with defective signal peptides. In such screens, it was noticed that the *prlC* (protein localization) mutation, which turned out to be a mutation in the *opdA* gene, could suppress the export defect of certain LamB signal sequence mutants (57). At present, it is not clear how mutations in OpdA can lead to the observed *prlC* phenotype.

Protease IV is highly conserved in eubacteria and archaea. In contrast, OpdA appears to be absent from gram-positive eubacteria and archaea. Like the protease IV of *E. coli*, its homologue in *B. subtilis*, denoted SppA (YteI), appears to be a membrane protein with three potential transmembrane segments. Disruption of the *sppA* gene of *B. subtilis* resulted in a decreased rate of processing of the α-amylase AmyQ but not of its translocation. This suggests that SPase I activity is negatively affected in the absence of SppA, for example, by the accumulation of certain signal peptides (30). Interestingly, *B. subtilis* contains a second gene (*tepA* or *ymfB*) for a potential protease with amino acid sequence similarity to SppA. In the absence of this so-called TepA protein (translocation-enhancing protein), the rate of translocation of a number of secretory proteins was strongly affected. Notably, the TepA protein, which probably has a cytoplasmic localization, also shows sequence similarity to the cytoplasmic protease ClpP (see below). Three possible roles of TepA in protein translocation have been suggested (30). First, TepA might be an analogue of *E. coli* OpdA, an idea which is based on the observation that signal peptides can inhibit protein translocation (62). Second, similar to what was observed for ClpP (176), TepA might have a regulatory function. Third, TepA might be a secretion-specific chaperone.

Extracytoplasmic Folding Catalysts

After leaving the translocation channel, secretory proteins have to fold into their native conformation at the *trans* side of the membrane. Since several proteases are present in this environment, rapid and correct folding is essential, in particular because (partly) unfolded proteins are very sensitive to proteases. This phenomenon is illustrated by the recent observation that an α-amylase from *B. licheniformis* (AmyL) was degraded when secreted by *B. subtilis* due to its relatively slow rate of folding. However, once folded into the native conformation, AmyL was stable in the growth medium (274). Folding at the *trans* side of the membrane is mediated by several extracellular folding catalysts, also termed foldases. In eubacteria, the foldases found thus far are PPIases, which catalyze the *cis-trans* isomerization of peptidyl-prolyl bonds, and thiol-disulfide oxidoreductases, which catalyze the formation and/or isomerization of disulfide bonds. In addition, other factors, such as certain cations, play a role in protein folding after translocation. Strictly speaking, the latter factors are not real components of the secretion machinery, but they can be regarded as such because they play an important role in the folding of secretory proteins of *B. subtilis*. Therefore, they are included in this overview.

PPIases. One of the proteins involved in the folding of proteins after their translocation is PrsA, a lipoprotein that is anchored to the outer leaflet of the cytoplasmic membrane (144, 145). Based on sequence similarity, it was proposed that PrsA is a PPIase belonging to the parvulin family (228). PrsA is essential for viability, and strains containing mutant PrsA proteins were shown to secrete lower amounts of degradative enzymes, probably due to decreased stability, resulting in increased sensitivity to proteolysis of these exoproteins (130, 144, 145). *E. coli* contains four periplasmic PPIases (68, 174), denoted RotA (cyclophilin type), FkpA (FKBP type), and SurA and PpiD (parvulin type). Moreover, *S. cerevisiae* produces five PPIases (76), denoted CPR2, CPR4, CPR5, CPR8, (cyclophilin type), and FPR2 (FKBP type), which are either localized in the ER or secreted. In addition, *S. cerevisiae* contains eight other PPIases that are localized in the nucleus, the mitochondrion, or the cytoplasm (76). Interestingly, extracytoplasmic PPIases of the cyclophilin and FKBP types appear to be absent from *B. subtilis*.

Thiol-disulfide oxidoreductases. Disulfide bonds are essential for the activity and stability of several proteins. Such bonds form spontaneously in vitro, but this process is much slower and less effective than the formation of disulfide bonds in vivo, which is catalyzed by thiol-disulfide oxidoreductases (210). In *E. coli*, the formation of disulfide bonds takes place in the periplasm. Six components involved in disulfide bond formation have been identified: DsbA, DsbB, DsbC, DsbD, DsbE, and DsbG (5). The Bdb (Bacillus disulfide bond) protein from *Bacillus brevis* was, until recently, the only known thiol-disulfide oxidoreductase from a gram-positive eubacterium (129). The *bdb* gene can complement a mutation in the *E. coli dsbA* gene, indicating that in the latter organism Bdb is translocated to the periplasm. It has been suggested that, in *B. brevis*, Bdb is also translocated across the membrane and localized at the membrane-cell wall interface.

Most likely, *B. subtilis* secretes only a limited number of proteins containing disulfide bonds (31). On the other hand,

secreted proteins of eukaryotes often contain several disulfide bonds (133). Proteins containing several disulfide bonds, like the human serum albumin and the human pancreatic α-amylase, are secreted very poorly by *B. subtilis* (31, 248), which may be due to the formation of incorrect disulfide bonds or the lack of formation of such bonds. However, disulfide bonds in proteins secreted by *B. subtilis* can be formed properly. This is evident from the observation that human interleukin-3, containing one disulfide bond, can be secreted efficiently by *B. subtilis* (310). The same applies to an engineered neutral protease from *B. subtilis* that was stabilized to a great extent by the introduction of a disulfide bond (160), and the *E. coli* alkaline phosphatase (PhoA) protein, which contains two disulfide bonds (31). This suggests that, in *B. subtilis*, thiol-disulfide oxidoreductases are present at the *trans* side of the cytoplasmic membrane. Screening of the genome of *B. subtilis* revealed three genes for proteins with similarity to thiol-disulfide oxidoreductases (31). Disruption of two of these genes, called *bdbB* (*yolK*) and *bdbC* (*yvgU*), did indeed interfere with the efficient secretion of *E. coli* PhoA. These two genes encode putative membrane-bound thiol-disulfide oxidoreductases that are related to *E. coli* DsbB. Disruption of the *bdbC* gene had the strongest effect on PhoA secretion, which was reduced approximately 10-fold (as measured through PhoA activity). This gene was also shown to be involved in the folding of A13i-β-lactamase, a hybrid precursor that contains one disulfide bond. The third gene encoding a putative thiol-disulfide oxidoreductase in *B. subtilis* is *bdbA* (*yolI*). The deduced BdbA protein shows a high degree of sequence similarity to Bdb from *B. brevis* (129) and may be a secreted protein. Disruption of this gene did not affect the secretion of *E. coli* PhoA or A13i-β-lactamase (31).

Propeptides. Propeptides, which are commonly present in secretory proteins from *Bacillus* species, are stretches of amino acids located between the signal peptide and the mature part of the protein. In *B. subtilis*, their lengths vary from 8 amino acids (α-amylase) (285) to 194 amino acids (neutral protease NprE) (334). Whereas no clear function has been assigned to short propeptides, long propeptides, which are mainly found in certain proteases, have two important functions. First, they prevent the activation of proteases prior to their translocation (323). Second, propeptides catalyze the folding and activation of the proteases once they have been translocated. It was shown that certain propeptides are functional not only in *cis* but also in *trans*, demonstrating their role as a molecular endochaperone (127, 128, 337). In general, the long propeptides in proteases are removed autocatalytically after translocation.

Other folding catalysts. Some secretory proteins require cations, such as Fe^{3+} and Ca^{2+}, for folding. The secretion of *B. subtilis* levansucrase is stimulated by growth of the cells in medium containing high concentrations of Fe^{3+}, and in vitro refolding of *B. subtilis* levansucrase was greatly enhanced by this cation, even though Fe^{3+} is not bound to the mature protein (49). Furthermore, several extracellular proteins from *B. subtilis*, such as levansucrase, neutral protease, and α-amylase, are calcium-binding proteins, and they require Ca^{2+} for stability (212, 275, 313). Ca^{2+} is trapped in the *B. subtilis* cell wall, thereby creating a microenvironment that must play an important role in the late steps of secretion (211). Notably, the charge properties of the cell wall will also influence its interactions with secretory proteins, as a protein with overall positive charge can be retarded by the negatively charged polyanionic polymers in the wall (50). Indeed, in vitro folding assays with a derivative of an α-amylase from *B. licheniformis* having an increased net positive charge showed a decreased rate of folding in the presence of cell wall material which was not observed in the folding of wild-type α-amylase. In addition, the cell wall had the capacity to bind large amounts of the mutated α-amylase (275). The latter observations imply that the folding and subsequent secretion of heterologous proteins can, in principle, be improved by the removal of positively charged residues that are not required for their stability or biological function.

QUALITY CONTROL

As indicated in the first sections of this review, *Bacillus* species are prolific and commercially important producers of high-quality industrial enzymes and a few eukaryotic proteins, such as human interleukin-3. The high quality of these secreted proteins is, at least in part, due to the presence of cellular quality control systems that efficiently remove misfolded or incompletely synthesized proteins. Paradoxically, these quality control systems represent major bottlenecks for the production of many heterologous proteins at commercially significant concentrations, because their folding is usually inefficient (29).

One of the greatest problems is proteolytic degradation. Thus far, most attempts to find solutions were focused on the proteases that are secreted into the growth medium (263). Mutants lacking up to six of these extracellular proteases have been made (75, 134, 329, 331). In some cases, these mutant strains were able to secrete increased amounts of (heterologous) proteins (29). Nevertheless, the effects were only moderately positive, and some proteins, like OmpA of *E. coli*, were still degraded rapidly (170). This degradation is probably caused by membrane- or cell wall-associated proteases that are part of the quality control system for exported proteins. Indeed, one cell wall-bound protease, CWBP52 (encoded by the *wprA* gene) (163), was shown to be involved in the degradation of the α-amylase from *B. licheniformis* (274) and an unstable variant of the signal peptidase SipS (28). In addition to degradation by membrane- or wall-associated proteases, it is conceivable that the degradation of heterologous proteins occurs in the cytoplasm.

Several cytoplasmic, membrane-bound, and extracytoplasmic proteases of *B. subtilis* have been identified through genetic and biochemical analysis, but the availability of the complete genome sequence of *B. subtilis* (149) enabled a systematic evaluation of a large number of (putative) proteases (Table 8). Several cytoplasmic proteases, such as ClpP, ClpQ, and Lon, are probably mainly involved in general housekeeping functions, such as degradation of misfolded proteins. However, some of these may also have more specific functions in gene regulation through proteolysis, as it was recently shown that ClpP-mediated proteolysis is involved in controlling the levels of MecA, a negative regulator of competence (176). The MlpA protein, which is highly similar to mitochondrial processing peptidases, appeared to have a role in gene regulation through proteolytic processing. The latter conclusion is based on the observation that in a strain lacking a functional *mlpA* gene, expression of the subtilisin gene (*aprE*) was increased about fivefold (32). Furthermore, the intriguing possibility was recently put forward that some membrane proteins or even secretory proteins of *B. subtilis* could be degraded by cytosolic proteases after their translocation, which would require their retrograde transport to the cytoplasm (28). Even though there is no direct evidence for retrograde transport in *B. subtilis*, this phenomenon has been documented for the degradation of certain ER-luminal proteins (19, 146, 214, 326). The latter proteins were shown to be returned to the cytoplasm via the Sec61p complex and degraded by the proteasome, a multimeric complex that is responsible for much of the proteolysis within the cytoplasm of eukaryotic cells (118).

Three homologues of the HtrA protein from *E. coli* were identified in *B. subtilis*. HtrA of *E. coli* is a periplasmic heat

shock protease involved in the removal of misfolded proteins from the periplasm (174). The *B. subtilis* HtrA homologues are encoded by the *htrA*, *yvtA*, and *yyxA* genes (for the correct sequence of YvtA, see D. Noone and K. M. Devine, GenBank accession no. AAF03153). These three genes are predicted to encode membrane-anchored proteins with their active sites located at the *trans* side of the membrane. Presently, the role of the HtrA homologues in *B. subtilis* is not known.

Several other proteases are present in the membrane that, most likely, have a quality control function for membrane-bound proteins. FtsH is a zinc-binding metalloprotease with its active site on the cytoplasmic side of the membrane. In *B. subtilis*, disruption of the *ftsH* gene resulted in a very pleiotropic phenotype (filamentous growth, sensitivity to heat and salt stress, and low levels of protein secretion) (71, 72). In *E. coli*, FtsH selectively degrades SecY when the latter protein is not in a complex with SecE, indicating that FtsH is indeed involved in quality control of proteins in the membrane (2). Notably, it was recently suggested that FtsH of *E. coli* could be involved in retrograde transport of membrane proteins and their degradation in the cytoplasm (136).

The type I SPases SipS, SipT, SipU, SipV, and SipW and the type II SPase are specifically involved in the cleavage of signal peptides from secretory preproteins. However, it cannot be excluded that these peptidases, in addition to preproteins, have other substrates. For example, it was demonstrated that the homologous Sec11p subunit of the SPase complex in the yeast ER membrane is involved in protein degradation (180).

CtpA and YvjB of *B. subtilis* are highly similar to the *E. coli* Tsp protein that is involved in the degradation of peptide-tagged proteins derived from truncated mRNAs. Keiler and coworkers (135) proposed a model in which truncated mRNAs that lack a stop codon are modified by the carboxyl-terminal addition of a peptide tag encoded by the SsrA RNA. This peptide tag is recognized by proteases, such as Tsp, that subsequently degrade the protein. Both CtpA and YjvB are predicted to remain attached to the membrane via an amino-terminal membrane anchor. The role of the two Tsp homologues and the SsrA RNA of *B. subtilis* in protein secretion or degradation has not yet been evaluated.

Finally, the YhfN protein, which is predicted to have seven membrane-spanning domains, shows sequence similarity to the Ste24 protease of *S. cerevisiae*, which is involved in the maturation of the α-factor, a small secreted pheromone (286). The function of YhfN is currently not known.

Sec-INDEPENDENT PROTEIN EXPORT

Although the Sec-dependent pathway seems to be responsible for the export of most proteins in *B. subtilis*, at least three alternative pathways seem to be present for the transport of small groups of specific proteins. These are a putative twin-arginine translocation (Tat) pathway, a prepilin-specific secretion and assembly pathway, and at least three ABC transporter-dependent secretion pathways. In the following sections we will discuss each of these three types of pathways.

A Twin-Arginine Translocation Pathway?

Like most other eubacteria, *B. subtilis* seems to contain a very recently identified Sec-independent secretion pathway, which is known as the Tat pathway. This pathway was first discovered in chloroplasts, in which it is involved in ΔpH-dependent protein import into the thylakoid lumen (47, 195, 232, 259). For the chloroplast system, it was shown that, in contrast to Sec-dependent translocation, proteins can be translocated in a folded conformation via this pathway (56, 58, 124, 233). Furthermore, it was demonstrated that two adjacent arginines combined with a hydrophobic determinant (preferably leucine) at position +2 or +3, relative to the twin arginines, were needed in the N-domain of signal peptides to direct precursors into this pathway (38, 39). In *E. coli*, precursors of several periplasmic cofactor (e.g., flavins, molybdopterins, and iron-sulfur clusters)-binding proteins have signal peptides carrying a similar RR-motif ([S/T]-R-R-x-F-L-K) immediately before their H-domain (18). Although the exact mechanism of protein export via the Tat pathway has yet to be unraveled, five components of the Tat pathway of *E. coli* have been identified. These are TatA (a putative membrane-bound receptor, homologous to the maize Hcf106 protein) (259), TatB (a TatA paralogue) (247), TatC (the putative translocase), TatD (a predicted soluble protein), and TatE (a TatA paralogue). These proteins are encoded by the *tatABCD* operon and the unlinked *tatE* gene (65, 246, 325). Disruption of the *tatA* to *tatC* and *tatE* genes affected the export of several preproteins with an RR-signal peptide, such as the trimethylamine *N*-oxide reductase TorA, which are transported in a Sec-independent manner (24, 245). The role of TatD, which is most likely a cytoplasmic protein, in the Tat pathway has not yet been established. Recent studies showed that a Sec-dependent periplasmic domain from the *E. coli* SPase I, also known as leader peptidase (lep), can be rerouted into the Tat pathway by the RR-signal peptide of TorA. In contrast, a full-length TorA-Lep fusion protein was not rerouted into the Tat pathway. Furthermore, it was shown that the TorA signal peptide could be converted into a Sec-targeting signal peptide by increasing the length and hydrophobicity of its H-domain (60). These findings indicate that the Sec and Tat pathways compete for preproteins, at least in *E. coli*, and that the overall hydrophobicity of the RR-signal peptide plays an important role in discrimination between these two pathways. Interestingly, *B. subtilis* contains three homologues of TatA/B/E (encoded by the *ydiI*, *yczB*, and *ynzA* genes), two homologues of TatC (encoded by the *ydiJ* and *ycbT* genes), and one TatD homologue (encoded by the *yabD* gene). Furthermore, at least one protein of *B. subtilis* with a putative RR-signal peptide is known to bind a cofactor. This is the QcrA protein, which contains an iron-sulfur cluster. It will be a major challenge for future research to determine the Tat-dependent exported fraction of the secretome.

Type IV Pilin Export

A second class of *B. subtilis* proteins that are exported in a Sec-independent manner consist of type IV pilin-like proteins encoded by the *comGC*, *comGD*, *comGE*, and *comGG* genes. The corresponding gene products are involved in the development of genetic competence. They resemble type IV pilins of various gram-negative eubacteria that are synthesized as precursors with cleavable signal peptides. Although prepilin signal peptides show certain similarities to signal peptides of secretory proteins and lipoproteins, the prepilin(-like) precursors are believed to bypass the Sec and Tat secretion pathways, as their translocation is dependent on a cleavage event at the cytoplasmic side of the membrane (54, 157, 199, 225, 226). ComC, the SPase that cleaves the *comG* products ComGF (an integral membrane protein) and ComGA (a putative ATPase located at the cytoplasmic side of the membrane), is known to be involved in the assembly of the pilin-like ComG proteins (54). The *B. subtilis* SPase ComC is an integral membrane protein with eight (putative) transmembrane regions, and this protein shows a high degree of similarity to prepilin peptidases of various other organisms (157). Processing of the *comG*

TABLE 8. Putative proteases and peptidases of *B. subtilis*

Protease[a]	Description	Location	Reference or SubtiList accession no.
AlbE (YwhO)	Similar to *Synechocystis* sp. processing peptidase	Cytoplasm	336
AlbF (YwhN)	Similar to mitochondrial zinc-endoprotease	Cytoplasm	336
AmpS	Putative aminopeptidase; metalloprotease	Cytoplasm	BG10986
AprX	Alkaline serine protease	Cytoplasm	BG12567
ClpE	ATP-dependent Clp protease-like	Cytoplasm	BG12578
ClpP	ATP-dependent Clp protease proteolytic subunit; requires ATP-binding subunit ClpA/ClpX	Cytoplasm	315
HslV (ClpQ, CodW)	Similar to β-subunit of the 20S proteasome; requires ATP-binding subunit HslU (ClpY)	Cytoplasm	265
IspA	Major intracellular serine protease	Cytoplasm	143
LonA	Heat shock ATP-dependent protease	Cytoplasm	230
LonB	Lon-like ATP-dependent protease	Cytoplasm	230
Map (AmpM)	Methionine aminopeptidase	Cytoplasm	185
MlpA (YmxG)	Similar to mitochondrial processing peptidase (metalloprotease)	Cytoplasm	32
Pcp	Pyrrolidone carboxyl peptidase	Cytoplasm	7
PepT	Tripeptidase	Cytoplasm	255
SpoVK	ATP-dependent FtsH-like protease	Cytoplasm	BG11039
TepA	Similarity to both SPPase and ClpP-type proteases	Cytoplasm	30
YclE	Similar to prolyl aminopeptidase	Cytoplasm	BG12026
YflG	Paralogue of methionine aminopeptidase (Map)	Cytoplasm	BG12942
YjbG	Similar to oligoendopeptidase	Cytoplasm	BG13136
YjoB	ATP-dependent FtsH-like protease	Cytoplasm	BG13216
YkvY	Similar to Xaa-Pro dipeptidase	Cytoplasm	BG13326
YlbL	Similar to Lon proteases	Cytoplasm	BG13364
YmfG	Similar to processing peptidase, paralogue of AlbE	Cytoplasm	BG13427
YmfH	Similar to mitochondrial processing peptidase (metalloprotease)	Cytoplasm	BG13428
YpwA	Similar to carboxypeptidase; metalloprotease	Cytoplasm	BG11458
YqgT	Similar to gamma-D-glutamyl-L-diamino acid endopeptidase I	Cytoplasm	BG11687
YqhT	Similar to Xaa-Pro dipeptidase	Cytoplasm	BG11708
YqjE	Similar to tripeptidase; paralogue of PepT	Cytoplasm	BG11734
YrrN	Putative protease; similar to collagenase from *Methanobacterium thermoautotrophicum*	Cytoplasm	BG13795
YrrO	Putative protease; paralogue of YrrN	Cytoplasm	BG13796
YtjP	Similar to Xaa-His dipeptidase	Cytoplasm	BG13867
YuiE	Similar to leucyl aminopeptidase	Cytoplasm	BG13970
YusX	Similar to oligoendopeptidase	Cytoplasm	BG14036
YwpE	Similar to surface protein sorting sulfhydryl protease (*Staphylococcus aureus* SrtA)	Cytoplasm	BG12499
YuxL	Similar to acylaminoacyl-peptidase	Cytoplasm	BG10463
ComC	Type IV prepilin-like SPase	Membrane	53
CtpA	Similar to carboxy-terminal processing protease (*E. coli* Tsp)	Membrane	162
FtsH	ATP-dependent zinc metallopeptidase	Membrane	72
HtrA (YkdA)	Similar to serine protease HtrA	Membrane	BG12608
Lsp	Type II SPase	Membrane	223
SipS/T/U/V/W	Type I SPases	Membrane	289, 290, 307
SppA (YteI)	SPPase	Membrane	30
YhcS	Similar to surface protein sorting sulfhydryl protease (*Staphylococcus aureus* SrtA)	Membrane	BG11597
YcdD	Similar to L-alanoyl-D-glutamate peptidase; putative lipoprotein	Membrane	BG12760
YhfN (YzoA)	Similar to prenyl protein-specific endoprotease from *S. cerevisiae*; putative metalloprotease	Membrane	BG11029
YvjB	Carboxy-terminal processing protease, similar to *E. coli* Tsp	Membrane	BG14110
YvtA	Similar to serine protease HtrA from *E. coli*	Membrane	Noone and Devine, GenBank accession no. AAF03153
YyxA (YycK)	Similar to serine protease HtrA from *E. coli*	Membrane	45
YodJ	Similar to D-alanyl-D-alanine carboxypeptidase	Cell wall	BG13538
DacA	Penicillin-binding protein 5 (D-alanyl-D-alanine carboxypeptidase)	Cell wall	324
DacB	Penicillin-binding protein 5* (D-alanyl-D-alanine carboxypeptidase)	Cell wall	43
DacF	Penicillin-binding protein (D-alanyl-D-alanine carboxypeptidase)	Cell wall	330
LytE	Cell wall hydrolase; probable endopeptidase	Cell wall	164
WprA	Cell wall-associated protein precursor (serine protease)	Cell wall	163
AprE	Alkaline serine protease (subtilisin E)	Extracellular	273
Bpr (Bpf)	Bacillopeptidase F (serine protease)	Extracellular	267
Epr (Ipa-15r)	Minor serine protease	Extracellular	266
Mpr	"Metalloprotease," but belongs to the family of serine proteases	Extracellular	267
NprB	Neutral protease B (metalloprotease)	Extracellular	297
NprE	Neutral protease E (metalloprotease)	Extracellular	334
Vpr (Ipa-45r)	Minor serine protease	Extracellular	268
YwaD (Ipa-8r)	Similar to aminopeptidase	Extracellular	BG10554

[a] Synonymous names are in parentheses.
[b] Accessible through http://bioweb.pasteur.fr/GenoList/SubtiList; BG codes are those used in SubtiList.

products is required for the assembly and anchoring of the pilin-like structures to the membrane, which in turn is required for DNA binding during transformation in *B. subtilis* (55, 80). ComC-like SPases cleave the peptide bond between a glycine at position −1 and a phenylalanine at position +1 (Fig. 2). Notably, the amino acid at position +1 relative to the SPase cleavage site of these pilins is modified, like the +1 cysteine residue of mature lipoproteins. However, in the case of type IV pilins, the phenylalanine residue at the +1 position is aminomethylated (157). A second difference with lipoprotein processing is that ComC is bifunctional, being responsible for both prepilin processing and the subsequent methylation of the +1 phenylalanine residue. The cleavage site of prepilins is located amino-terminal to the hydrophobic H-domain (225). Consistent with the latter observation, the putative active site of prepilin SPases appears to be localized in the cytoplasm (151). Interestingly, even though prepilin SPases and type II SPases lack sequence similarity, the catalytic mechanism of prepilin SPases seems to be related to that of type II SPases, as the potential active sites of both SPases contain two catalytic aspartic acid residues. Furthermore, in both types of SPases, the potential active-site residues are predicted to reside in close proximity to the membrane surface. However, the putative catalytic aspartic acid residues of type IV prepilin SPases are located at the cytoplasmic side of the membrane, while those of type II SPases are located at the extracytoplasmic side (151, 293).

Export via ABC Transporters

Several *Bacillus* species produce peptide antibiotics which are synthesized through either a ribosomal or nonribosomal mechanism (81, 99, 189). Some of the ribosomally synthesized antimicrobial peptides contain signal peptides for their translocation across the membrane by dedicated ABC transporters (70, 112, 239). In *B. subtilis* 168, the *sunS-sunT* operon has recently been shown to encode, respectively, the lantibiotic sublancin 168 and the ABC transporter SunT, which is required for sublancin production (203). Mature sublancin 168 contains one β-methyllanthionine bond and two disulfide bonds. Like signal peptides of other lantibiotics (48, 192), the sublancin signal peptide contains a double glycine motif amino-terminal to the processing site. Interestingly, ABC transporters such as SunT have a dual role in secretion, as they are responsible both for removal of the signal peptide and for translocation of the mature lantibiotic across the cytoplasmic membrane. The protease domain of these so-called dual-function transporters is localized in their conserved amino terminus, which contains cysteine and histidine residues involved in precursor cleavage (98, 112, 314). A second lantibiotic known as subtilin is secreted by the *B. subtilis* strain ATCC 6633. This lantibiotic contains a signal peptide that is unrelated to those of sublancin and the bacteriocin subtilosin (see below). Nevertheless, subtilin is secreted by a dedicated ABC transporter, like sublancin and subtilosin (11, 52, 138).

Similar to sublancin and subtilin, the antilisterial bacteriocin subtilosin is ribosomally synthesized as a precursor; it then matures and is exported as a cyclic peptide containing several modifications, including one disulfide bridge. Notably, presubtilosin lacks the sequence motifs known to be required for prelantibiotic processing, as found in sublancin (336). The downstream operon of the *sbo* gene coding for subtilosin contains seven genes, *albA* to *albG* (*ywiA* to *ywhM*). This operon was shown to be essential for subtilosin production. Genes in the *alb* operon encode proteins, such as an ABC transporter (AlbC) and two peptidases (AlbE and AlbF), which are likely to be required for the processing and export of subtilosin (336). Interestingly, the *albA* gene product is homologous to proteins involved in cofactor synthesis (e.g., molybdenum cofactors) (336), while the *albB* (*ywhR*) gene encodes a putative protein with an RR-signal peptide (Table 3). Thus, it is conceivable that AlbB is exported via the Tat pathway, which in gram-negative eubacteria, is known to be required for the export of cofactor-binding proteins (18, 245).

Finally, the extracellular pheromone ComX, which is involved in cell density-controlled onset of the transcription of competence genes, is also ribosomally synthesized as a precursor and modified prior to secretion (159, 272). Although not documented, it is conceivable that a dedicated ABC transporter is responsible for the processing and secretion of this pheromone.

CELL WALL RETENTION

The cell wall of *B. subtilis* defines a cellular compartment containing approximately 9% of the total cellular protein content, analogous to the gram-negative periplasm (217). In *B. subtilis*, proteins retained in the cell wall include DNases, RNases (173), proteases (10, 163, 274), enzymes involved in the synthesis of peptidoglycan (penicillin-binding proteins), and cell wall hydrolases (20, 96, 97) that are involved in cell wall turnover during cell growth, cell division, sporulation, and germination (181, 182, 219, 335).

Cell Wall Retention Signals

Several *B. subtilis* enzymes involved in cell wall turnover contain a variable number of repeated domains which have affinity for components of the cell wall (102, 164, 229). These repeats are thought to direct enzymes for cell wall assembly and turnover to specific sites where cell wall synthesis and/or hydrolysis takes place, as was shown for *Staphylococcus aureus* (8, 9). Most likely, this specific targeting is promoted by certain components of the cell wall, such as choline, which was shown to be a receptor for several cell wall proteins of *Streptococcus pneumoniae* (234, 240, 241). Like the enzymes involved in cell wall turnover, other *B. subtilis* proteins also retained in the cell wall, such as WprA and WapA, contain repeated motifs for cell wall binding. At present it is not known which receptors are involved in directed targeting of cell wall proteins in *B. subtilis*. The mature parts of 16 of the proteins with cleavable signal peptides (Tables 1 and 3) contain (putative) cell wall-binding repeats, indicating that they may be retained in the cell wall. Surprisingly, potential cell wall-binding regions also might be present in some (predicted) membrane proteins, such as HtrA, YclI, and YxcE, suggesting that these proteins are active at sites of intimate contact between the membrane and the cell wall.

Covalent Attachment to the Cell Wall?

A second group of surface proteins of gram-positive organisms are covalently anchored to the cell wall via the carboxyl terminus. The amino-terminal domains of these molecules are displayed on the eubacterial surface (252, 253). Cell wall anchoring of surface proteins in *S. aureus* requires, in addition to the amino-terminal signal peptide, a carboxyl-terminal cell wall sorting signal consisting of the so-called LPxTG motif, a carboxyl-terminal hydrophobic domain, and a positively charged tail (190, 191, 254). During the export of surface proteins, the cell wall sorting signal is cleaved between the Thr and Gly residues of the LPxTG motif by the so-called sortase (SrtA) (169). Simultaneously, the carboxyl group of the Thr residue is linked to the cell wall by a branched anchor peptide (295).

In contrast to *S. aureus*, none of the putative exported *B. subtilis* proteins contains a carboxyl-terminal LPxTG reten-

tion signal for covalent attachment to the cell wall. Nevertheless, genes for two SrtA homologues could be identified in the *B. subtilis* genome. First, the *yhcS* gene codes for a putative exported protein of 198 amino acids with an amino-terminal membrane anchor showing 22% sequence identity to SrtA of *S. aureus*. This protein contains the carboxyl-terminal LxTC motif, of which the cysteine is essential for SrtA activity (296). Second, a small open reading frame called *ywpE* codes for a protein of 102 amino acids that also contains the LxTC motif, showing 23% sequence identity with the carboxyl terminus of SrtA. Notably, the latter protein is predicted to be a cytoplasmic protein. Taken together, these findings suggest that at least one sortase-like enzyme for the cleavage and linkage of surface proteins is present in *B. subtilis*. If so, this enzymes recognizes amino acid sequences that are different from the LPxTG motif of *S. aureus* surface proteins. Alternatively, the sortase-like proteins of *B. subtilis* may be involved in completely different, as yet unidentified, processing events.

SPORULATION-SPECIFIC PROTEIN TRANSPORT

In response to nutrient starvation, *B. subtilis* develops endospores to resist a variety of harsh conditions, such as extreme cold or heat (279). At the beginning of the sporulation process, an asymmetrically positioned septum is formed that divides the cell into two unequally sized compartments. Subsequently, the larger compartment (the mother cell) engulfs the smaller compartment (the forespore), which ultimately becomes the spore. Thus, the forespore is surrounded by two membranes. The IMS between these two membranes is the assembly site of two layers of specialized peptidoglycan, called the germ cell wall and the cortex. Consequently, proteins residing in the germ cell wall or cortex must be sorted to the IMS between the forespore and the mother cell. This subcellular compartmentalization imposes a requirement for intracellular protein sorting on the cells, because protein synthesis is limited to the cytoplasm of the mother cell and the forespore.

Spore Protein Traffic

One of the processes that requires protein transport during sporulation is the communication between the mother cell and the forespore. Two proteins were shown to be exported by the forespore and to interact with membrane proteins of the mother cell. First, the SpoIIR protein, synthesized in the forespore prior to engulfment, contains a functional signal peptide which can drive the export of the mature part of the protein. The mature SpoIIR protein is thought to activate, directly or indirectly, the receptor/protease SpoIIGA, which is required for pro-σ^E processing (120, 121). Second, the SpoIVB protein is synthesized in the forespore and transported across the forespore inner membrane after engulfment has taken place. SpoIVB probably remains anchored to the latter membrane, but a smaller form seems to be released into the IMS of the spore, allowing the activation of receptors and proteases in the outer forespore membrane that are responsible for pro-σ^K processing (63). As the amino terminus of SpoIVB contains a putative signal peptide but lacks a putative SPase I cleavage site, it is presently unclear which protease is responsible for the processing and subsequent release of SpoIVB in the IMS of the forespore. Other processes in sporulation which require transport of proteins are the biogenesis of the germ wall and spore-cortex in the IMS of the forespore and the degradation of the spore peptidoglycan during germination. CwlD and DacB (also known as PBP5*) (218, 220) are the only proteins with a putative signal peptide that were reported to be involved in cortex synthesis. However, the precise subcellular localization of DacB and CwlD has not yet been documented. The germination-specific amidase SleB was found to be localized on the exterior side of the cortex in spores, while its synthesis is forespore specific (175). The fact that pre-SleB has to be transported across the forespore inner membrane and processed into its mature form to reach the IMS implies that a functional protein translocation machinery and at least one of the type I SPases are present in the forespore inner membrane. Other proteins involved in spore-cortex synthesis, such as SpoVB and SpoVE (279), are predicted to be transmembrane proteins with loops exposed in the IMS of the forespore. Finally, the recent finding that TasA (for translocated antibacterial spore-associated protein), a protein with a broad spectrum of antibacterial activity, is transported to *B. subtilis* endospores provides another example of spore-specific protein sorting. TasA is thought to confer a competitive advantage to the spore during the onset of sporulation and later, during germination, by inhibiting the growth of other organisms (276). In addition, TasA has been suggested to be required for proper spore coat assembly (258).

Factors Involved in Spore Protein Traffic

Although not much is known about the factors involved in protein transport from one sporulation-specific compartment to another, it is conceivable that certain factors involved in the secretion of proteins by vegetative cells are also involved in the sorting of proteins, such as TasA and SleB, to the IMS of forespores. Three lines of evidence indicate that this is indeed the case. First, SecA is likely to be involved, directly or indirectly via the secretion of certain Phr peptides, in some of these sorting events, as *divA* mutant strains are defective in sporulation (6, 238, 284). Second, a requirement for the type I SPases SipT and SipV for sporulation was recently reported (132). Finally, recent studies to identify determinants of the subcellular sorting of TasA showed that SipW is required for this process (258, 276, 293a).

PERSPECTIVES

As described in this review, important progress has been made in characterization of the protein transport machineries of *B. subtilis*, the Sec machinery in particular, since the complete genome sequence of this versatile and amenable organism was published by Kunst et al. in 1997 (149). The present "genome-based" predictions concerning the various types of signal peptides that direct protein transport in *B. subtilis* have fruitfully built upon these sequencing data and will, hopefully, form a solid basis for further studies leading to a full definition of the secretome. Major challenges for future research are provided by the recent identification of alternative Sec-independent pathways for protein transport, such as the Tat pathway. First, it will be very important to identify the critical components of these novel pathways. This is of particular relevance in the case of the protein transport pathways facilitating spore protein traffic, of which close to nothing is presently known. Second, and perhaps even more interesting, the factors that direct different exported proteins into distinct pathways for protein transport in *B. subtilis*, for example the Sec or the Tat pathway, need to be defined. Finally, from the applied point of view, it will be of particular importance to unravel the mechanisms that the *Bacillus* cell uses for optimization of its specificity and capacity for protein secretion, the folding of secreted proteins, and their quality control. The fact that *B. subtilis* can modulate its capacity and specificity for protein

secretion through temporally controlled expression of the gene (*sipS*) for a secretion pathway component was first documented by Bolhuis et al. in 1996 (26). Strikingly, those studies showed that the *sipS* gene was expressed in concert with the genes for degradative enzymes, its transcription being under the control of the DegS-DegU two-component regulatory system, which is one of the major regulatory systems involved in the synthesis of degradative enzymes in the postexponential growth phase (95). In the meantime, it has been shown that the expression of genes for other secretion pathway components, such as SecA (116), SecDF (27), SipP (292), SipT (289, 290), SipW (276, 277), SPase II (291), SppA (30), BdbA, and BdbB (31), is regulated in a growth phase-dependent manner and/or in response to changes in the environment. In contrast, the genes for other components, such as TepA (30), SipU, SipV (289, 290), and BdbC (31) appeared to be expressed constitutively. Strikingly, the expression patterns for many of these genes show considerable differences (31). It seems likely that the different patterns reflect different responses of the cell to prevent potentially detrimental situations that can be caused by high-level protein secretion, but the molecular basis for such responses is presently not clearly understood. The implementation of advanced technologies, including DNA array and proteome-secretome analyses, will be required to evaluate the biological relevance of the latter observations and, ultimately, to exploit the natural protection mechanisms against "secretion stress" for the high-level production of an extended range of proteins of commercial value by the *B. subtilis* cell factory. These are major aims of our ongoing research that is carried out in close collaboration with partners in the so-called European *Bacillus* Secretion Group.

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